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Ingénierie tissulaire de la pulpe dentaire : vers le développement d'un médicament de thérapie innovante

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Liste des abréviations

AAE : Association Américaine des Endodontistes (*American Association of Endodontists*)

AMM : Autorisation de Mise sur le Marché

ANSM : Agence Nationale de Sécurité du Médicament

ATMP : Produit Médicinal de Thérapie Avancée (*Advanced-Therapy Medicinal Product*)

BMP : Protéine de Morphogenèse Osseuse (*Bone Morphogenetic Protein*)

hBMP-7 : Protéine de Morphogenèse Osseuse humaine-7 (*human Bone Morphogenetic Protein-7*)

BMSSC : Cellule Souche/Stromale de Moelle Osseuse (*Bone Marrow Stromal Stem Cells*)

BPF : Bonnes Pratiques de Fabrication

CATP : Produit de Thérapie Avancée Combiné (*Combined Advanced Therapy Product*)

CBMP : Produit Médicinal d'origine Cellulaire (*Cell-Based Medicinal Product*)

CD : Cluster de différenciation

CIP : Ciprofloxacine

Co-CS-HA : Collagène-Chondroïtine Sulfate-Acide Hyaluronique

Col : Collagène

CP : Poudre de Céramique (*Ceramic Powder*)

CPD : Cellule de Pulpe Dentaire

CSDP : « Culot cellulaire Amatriciel dérivé d'un Feuillet de Cellules Souches » (*Scaffold-free stem-Cell-Sheet-Derived Pellet*)

CSM : Cellule Souche/Stromale Mésenchymateuse

CSM-GW : Cellule Souche/Stromale Mésenchymateuse de Gelée de Wharton

CSM-PD : Cellule Souche/Stromale Mésenchymateuse de Pulpe dentaire

CTI : *Cell Therapy Institute*®

DDM : Matrice Dentinaire Déminéralisée (*Demineralized Dentin Matrix*)

DMP-1 : Protéine Matricielle Dentinaire-1 (*Dentin Matrix Protein-1*)

DOX : Doxycycline

EDTA : Acide Ethylène Diamine Tétra-acétique

Ef : *Enterococcus faecalis*

FDA : Office de contrôle Pharmaceutique et Alimentaire (*Food and Drug Administration*)

FGF : Facteur de Croissance Fibroblastique (*Fibroblast Growth Factor*)

G-CSF : Facteur Stimulant les Colonies de Granulocytes (*Granulocyte Colony-Stimulating Factor*)

GF : Facteur de Croissance (*Growth Factor*)

GTMP : Produit Médicinal de Thérapie Génique (*Gene Therapy Medicinal Product*)

H : Hydrogel

HNT : Nanotubes Halloysite (*Halloysite Nanotubes*)

HLA : Antigène Leucocytaire Humain (*Human Leukocyte Antigen*)

MDP : Peptide MultiDomaine (*MultiDomain Peptide*)

MEC : Matrice ExtraCellulaire

MET : Métronidazole

MTA® : Mineral Trioxide Aggregate®

MTI : Médicaments de Thérapie Innovante

MTI-PP : Médicaments de Thérapie Innovante Préparés Ponctuellement

NF : Nanofibreux (*Nanofibrous*)

nHA: nano-HydroxyApatite

OMS : Organisation Mondiale de la Santé

PA : Peptide Amphiphile

PCL : Poly(ε-CaproLactone)

PDS-II : Support nanocomposite composé de PolyDioxanone (*nanocomposite Scaffold composed of PolyDioxanone*)

PEG : PolyEthylène Glycol

Pg : *Porphyromonas gingivalis*

PLCL : Poly(L-lactide-co-ε-caprolactone)

PLGA : Acide Poly(Lactique-co-Glycolique) (*Poly[lactic-co-glycolic] Acid*)

PLLA : Acide Poly L-Lactique (*Poly(L-lactic acid]*)

PTC : Préparation de Thérapie Cellulaire

rhCol : Collagène humain recombinant (*recombinant human Collagen*)

sCTMP : Produit Médicinal de Thérapie Cellulaire somatique (*somatic Cell Therapy Medicinal Product*)

SDF-1 : Facteur Dérivé des cellules Stromales-1 (*Stromal cell-Derived Factor-1*)

SP : *Side Population*

TEP : Produit d'Ingénierie Tissulaire (*Tissue Engineered Product*)

PREAMBULE

La pratique de la médecine bucco-dentaire connait actuellement une vraie révolution grâce au développement de nouvelles notions comme, par exemple :

- La **dentisterie basée sur les faits** ou *Evidence-based dentistry*, qui impose une remise en question régulière pour offrir la meilleure thérapeutique à notre patient.
- Le **gradient thérapeutique**, qui reprend le *Primum non nocere* d'Hipprocrate et repousse les limites de notre pratique vers une dentisterie *a minima*, la moins invasive possible.
- Le **biomimétisme**, qui propose d'étudier le vivant pour développer des biomatériaux mimant de mieux en mieux les tissus de la dent.

Ainsi, à l'heure d'une dentisterie basée sur les faits, la dent doit être reconstruite *ad integrum*, en respectant un gradient thérapeutique et de manière biomimétique.

Cette approche a déjà fait beaucoup de chemin, car nous disposons aujourd'hui de biomatériaux adhésifs permettant de reconstruire « prophétiquement » la dentine et l'émail, avec des propriétés qui sont de plus en plus proches de celles des tissus naturels.

Cependant, la reconstruction de la pulpe dentaire apparait plus compliquée à réaliser, car contrairement à l'émail et la dentine, c'est un tissu qui contient des cellules. La stratégie actuelle de scellement de l'espace endodontique par compactage de gutta-percha est très éloignée du concept de biomimétisme, car l'absence de cellules au sein de ce matériau supprime la possibilité de percevoir les stimuli nociceptifs ainsi que le potentiel d'adaptation du complexe dentinopulpaire, notamment en cas de lésions carieuses. Cette situation n'est pas satisfaisante lorsque l'on sait que la gestion de l'endodonte conditionne le pronostic de nos restaurations prothétiques et donc celui de la dent.

Dans ce contexte, des stratégies thérapeutiques alternatives permettant de maintenir ou de retrouver la vitalité pulpaire sont nécessaires. Celles qui font appel à l'ingénierie tissulaire, qui propose d'incorporer des cellules souches/progénitrices dans un biomatériau pour reconstituer un tissu structurellement et fonctionnellement similaire à l'original, sont aujourd'hui particulièrement prometteuses.

C’est avec cette approche d’ingénierie tissulaire que nous avons réalisé ce travail, afin d’initier le développement d’un médicament de thérapie innovante permettant la régénération de la pulpe dentaire dans l’endodonte préalablement désinfecté et mis en forme.

Ce travail n’est certes que la première étape du développement de ce nouveau médicament, mais comme l’a dit John Lennon :

« A dream you dream alone is only a dream. A dream you dream together is reality ».

Alors j’espère qu’en lisant ce travail vous commencerez à croire comme moi en ce rêve de revitaliser les dents, pour peut-être qu’un jour, nous puissions faire de ce rêve une réalité.

INTRODUCTION

1. Composition et physiopathologie de la pulpe dentaire

1.1 Composition de la pulpe dentaire

La pulpe dentaire est le tissu conjonctif lâche situé au centre de la dent. Elle est presque totalement entourée par de la dentine (Figure 1), tissu minéralisé produit par des cellules hautement spécialisées, les odontoblastes, organisées en palissade à l'interface dentine-pulpe.

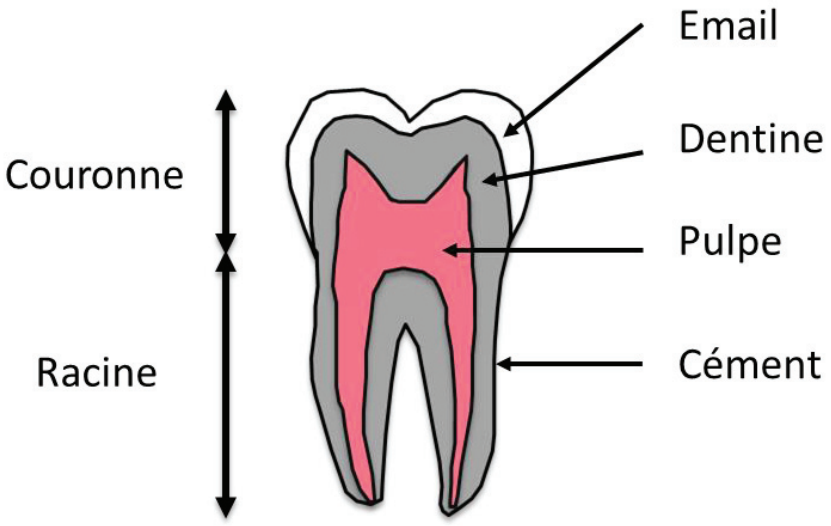

Figure 1 : Représentation schématique des différentes structures et tissus de la dent.

La pulpe dentaire a pour rôles principaux de maintenir l'homéostasie de la dent grâce à ses fonctions de nutrition de la dentine et de la pulpe, de perception des agressions que subit la dent, de défense du tissu pulpaire (agressé par exemple par des bactéries orales lors du processus carieux), et de réparation (par exemple lors des phénomènes de la cicatrisation dentinopulpaire). Elle contient différents types cellulaires dispersés à l'intérieur d'une matrice extracellulaire dense, dont les principaux sont (Figure 2) :

- des cellules endothéliales, des péricytes et des cellules musculaires lisses qui assurent la **vascularisation (cf. 1.1.2)** de la pulpe,
- des fibres nerveuses et des cellules associées qui permettent l'**innervation** du tissu **(cf. 1.1.3),**
- des **odontoblastes** qui fabriquent et déposent la dentine **(cf. 1.1.4.1),**

- des cellules **immunitaires** qui assurent la défense du tissu et des **fibroblastes** qui assurent le renouvellement de la matrice extracellulaire **(cf. 1.1.4.2),**
- des cellules **souches/progénitrices** mésenchymateuses (CSM) impliquées dans les phénomènes de régénération/réparation pulpodentinaire **(cf. 1.3).**

	Dentin	Pulp
Cells	Odontoblasts exclusively	Fibroblasts (pulpoblasts), vascular cells, pericytes, neural cells, histiocytes/ macrophages, dendritic cells, lymphocytes, mast cells
Collagens	Types I and I trimer (98%) Types III (1-2%) and V (1%) (90% of the dentin ECM)	Type I (56%) Types III (41%) and V (2%); Type VI (0.5%) associated with microfibrillin
Non-collagenous proteins	(10% of the dentin ECM)	
	Phosphorylated matrix proteins (SIBLINGs): DSPP > DSP and DPP DMP-1, BSP, OPN, MEPE	 none BSP, OPN
	Non-phosphorylated matrix proteins: Matrix GLA protein, osteocalcin, osteonectin	Fibronectin Osteonectin (in tooth germs)
	Proteoglycans (SLRPs) CS/DS PGs: decorin-biglycan (CS-4 81%, CS-6 14%, CS/DS 2%) KS PGs: lumican, fibromodulin, osteoadherin	Versican CS-4 and -6, 60%; DS, 34%; KS, 2% Hyaluronic acid
	Amelogenin 5-7 kDa Growth factors: TGF-β, ILGF-I and -II, FGF-2, VEGF, PDGF	 BMPs Types IA and II receptors for TGF-β, activin, and BMPs
	Metalloproteinases: collagenase (MMP-1), gelatinases (MMP-2 and -9), stromelysin-1 (MMP-3), enamelysin (MMP-20), MT1-MMP, TIMP-1 to -3	MMPs: collagenases, gelatinases, stromelysin-1 TIMPs
	Alkaline phosphatase Serum-derived proteins: αHS_2-glycoprotein, albumin, lipoproteins	 Fibronectin of serum origin
	Phospholipids: Membrane phospholipids (66%) Extracellular-mineral-associated phospholipids (33%)	 Membrane and ECM phospholipids

Figure 2 : Composition cellulaire et protéique de la dentine et de la pulpe dentaire humaine.
(Goldberg & Smith 2004)

1.1.1 La matrice extracellulaire (MEC)

La MEC pulpaire est composée principalement de collagènes de type I et III, et de types IV, V et VI en plus faible quantité (Goldberg et al. 2008)(Goldberg et al. 2011). Les collagènes pulpaires jouent un rôle structural de support comme dans les autres tissus conjonctifs. Les glycosaminoglycanes constituent plus de la moitié des molécules matricielles pulpaires. Ce sont essentiellement des chondroïtines sulfates, des dermatanes sulfates, des kératanes sulfates, des héparanes sulfates et de l'acide hyaluronique. Leur principale fonction est le maintien de l'hydratation du tissu. Les principales glycoprotéines retrouvées dans la pulpe sont la fibronectine, la ténascine et la thrombospondine. Elles jouent un rôle dans l'adhésion des fibroblastes pulpaires au réseau collagénique. La fibronectine aurait aussi un rôle dans le maintien de la morphologie des odontoblastes. Les métalloprotéases matricielles stockées dans la MEC pulpaire participent à la dégradation des protéines extracellulaires, permettant ainsi le remodelage du tissu et le déroulement correct des phénomènes inflammatoires et cicatriciels (Goldberg et al. 2009)(Bogović et al. 2011).

1.1.2 La vascularisation

La pulpe est un tissu richement vascularisé. Les vaisseaux sanguins pénètrent dans la pulpe par le(s) foramen(s) apical(aux), puis ils progressent au centre des canaux radiculaires en direction de la chambre pulpaire où ils se ramifient en artérioles qui se ramifient elles-mêmes pour former un vaste réseau de capillaires à la périphérie du tissu. Les capillaires pulpaires les plus périphériques facilitent la diffusion des nutriments vers les odontoblastes (Figure 3).

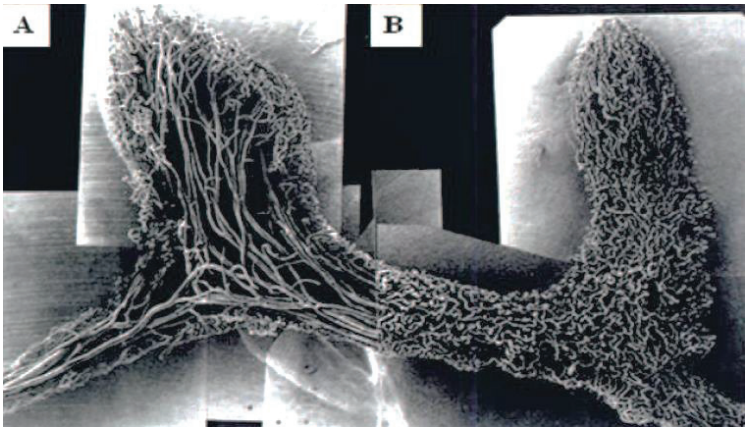

Figure 3 : Moulage en résine montrant la vascularisation d'une molaire. (A) Vascularisation périphérique. (B) Vaisseaux de la partie centrale de la pulpe et ramifications périphériques.
(Nanci 2008)

Le retour veineux se fait également par le foramen apical grâce à des veinules qui se regroupent pour former des veines collectrices qui cheminent au voisinage des artères dans la partie centrale du canal radiculaire et sortent de la pulpe par le foramen apical (Nanci 2008).

1.1.3 L'innervation

Le réseau nerveux pulpaire est essentiellement constitué de fibres sensitives issues du nerf trijumeau dont les corps cellulaires se trouvent dans le ganglion trigéminal. Les fibres nerveuses pénètrent dans la pulpe par le foramen apical et les canaux accessoires. Elles se regroupent au centre du canal radiculaire pour former de volumineux faisceaux nerveux qui se ramifient progressivement dans la chambre pulpaire et se terminent sous la forme d'un réseau dense de fibres nerveuses appelé plexus nerveux sous-odontoblastique (ou plexus de Raschkow) (Bletsa et al. 2009)(Magloire et al. 2009) (Couve et al. 2013). Certaines fibres nerveuses pénètrent dans la couche odontoblastique et la prédentine, puis dans les tubules dentinaires (Maeda et al. 1987). Les fibres nerveuses pulpaires sont pour la plupart des fibres amyéliniques de type C activées essentiellement au cours de l'inflammation pulpaire pour transmettre la douleur. La pulpe contient également des fibres A-δ impliquées également dans la transmission douloureuse. Ces fibres seraient stimulées par le déplacement du fluide dentinaire intratubulaire ou par un signal d'origine odontoblastique. Des fibres myéliniques A-β ont aussi été mises en évidence dans la pulpe. Elles pourraient être impliquées dans la transmission de sensations non douloureuses engendrées par des stimulations de très faible intensité, par exemple de type vibratoire (Piette 2001).

1.1.4 Les principales Cellules de la Pulpe Dentaire (CPD)

1.1.4.1 Les odontoblastes

A l'interface pulpe-dentine se trouvent les odontoblastes, cellules mésenchymateuses hautement différenciées issues des crêtes neurales et dont la fonction principale est la formation de la dentine. Les odontoblastes sont organisés en une couche jointive à la périphérie pulpaire. Ils émettent chacun un long prolongement cytoplasmique dans la dentine (Couve et al. 2013).

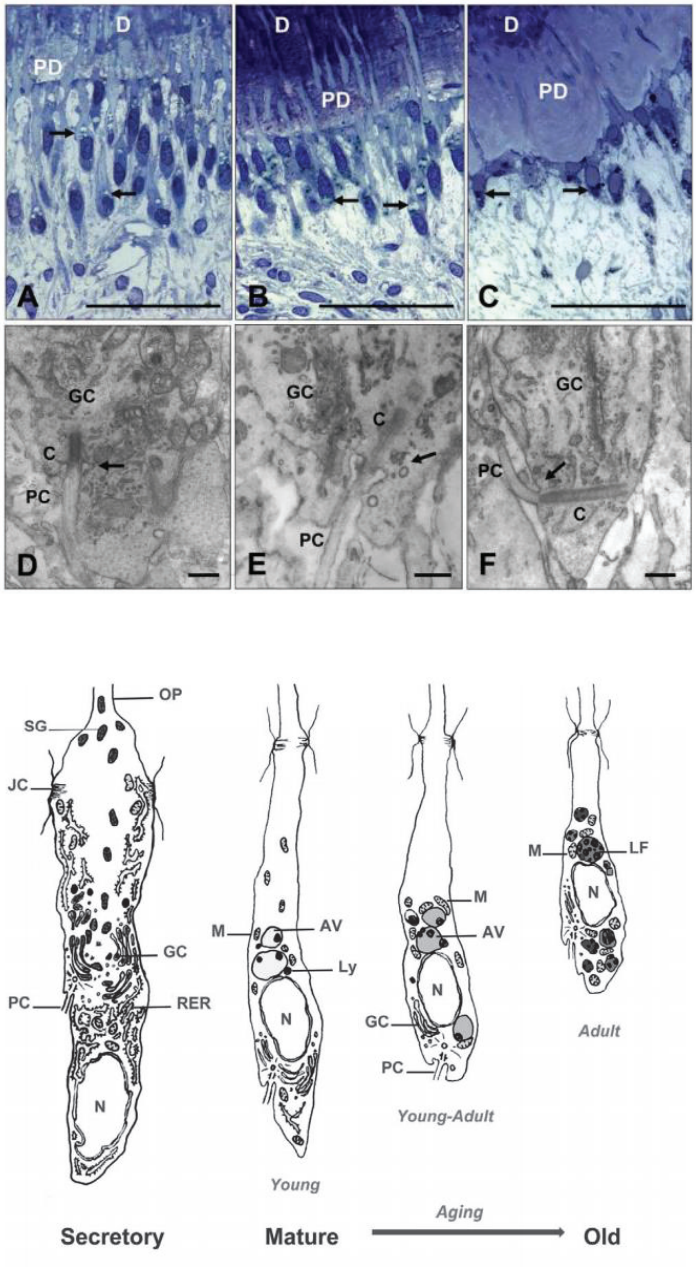

Figure 4 : L'odontoblaste à différents stades de maturation. L'odontoblaste va progressivement passer d'un stade sécrétoire très actif (A et D) à un stade mature où il est beaucoup moins actif (B et E). Il vieillit ensuite progressivement jusqu'à atteindre un stade de quiescence (C et F).
(Couve et al. 2013)

Une première synthèse de dentine a lieu lors de l'organogenèse de la dent avec un odontoblaste en phase sécrétoire (4 à 6 µm/jour), puis l'activité se réduit (0,5µm/jour) et la cellule devient quasiment quiescente (Figure 4)(Couve et al. 2013).

1.1.4.2 Les fibroblastes et les cellules immunitaires

Les fibroblastes pulpaires sont dispersés dans le tissu. Dans les conditions physiologiques, ils sont responsables de la formation et du renouvellement des composants de la matrice extracellulaire (Goldberg and Smith, 2004). Ils jouent également un rôle important dans la physiopathologie de l'organe, par exemple lors des phénomènes inflammatoires provoqués par la pénétration des bactéries cariogènes dans la dentine. En effet, ils modifient la structure de la MEC pour permettre la migration cellulaire.

Des cellules immunocompétentes, caractérisées par l'expression du récepteur CD45, sont présentes dans la pulpe dentaire saine où elles assurent l'immunosurveillance du tissu (Jontell et al. 1998). Parmi ces cellules, on distingue différentes sous-populations : les granulocytes (CD16+/CD14-), les lymphocytes T (CD45+/CD3+), les monocytes et les macrophages (CD45+/CD14+), les cellules dendritiques (CD45+/HLA-DR ^{hi}/ Lin1^{-}) et les cellules NK (*Natural Killer*) (CD45+/CD3-/CD56+) (Figure 5) (Gaudin et al. 2015).

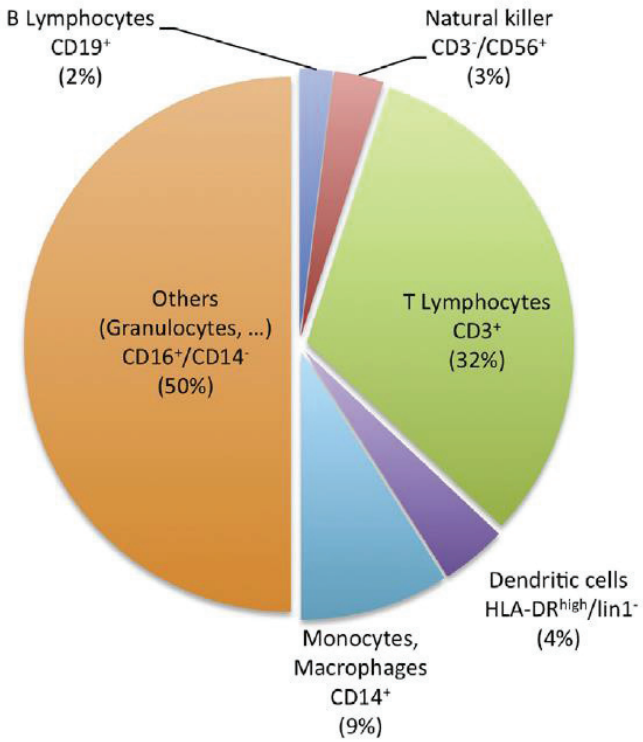

Figure 5 : Proportion des différentes populations de cellules immunocompétentes CD45+ dans la pulpe dentaire humaine saine. (Gaudin et al. 2015)

1.2 Organogenèse de la pulpe dentaire

1.2.1 Migration de cellules des crêtes neurales et odontogenèse

Le développement de l'organe dentaire débute suite à la migration de cellules ectomésenchymateuses issues des crêtes neurales céphaliques dans les bourgeons maxillaire et mandibulaire du premier arc branchial. Ces cellules vont induire l'invagination de l'ectoderme oral dans l'ectomésenchyme sous-jacent au niveau des sites où vont se former les dents. Cette invagination est appelée lame dentaire. Puis, grâce à une succession d'interactions séquentielles et réciproques entre les cellules ectomésenchymateuses et les cellules ectodermiques, les germes dentaires vont se former et acquérir progressivement une morphologie spécifique de chaque dent. Ils sont constitués principalement de la papille ectomésenchymateuse dentaire, qui va donner naissance à la pulpe dentaire et à la dentine, et de l'organe épithélial de l'émail qui va donner naissance à l'émail. A l'issue de cette phase de morphogenèse, des phénomènes hautement régulés de différenciation cellulaire vont conduire à l'apparition des odontoblastes, cellules qui produisent la dentine, à partir des cellules ectomésenchymateuses de la papille dentaire, et des améloblastes, cellules qui produisent l'émail, à partir des cellules ectodermiques de l'organe de l'émail.

1.2.2 Formation de la pulpe dentaire

La pulpe dentaire succède à la papille ectomésenchymateuse dentaire lorsque les premiers odontoblastes se différencient en périphérie de celle-ci. La différenciation odontoblastique est un mécanisme complexe régulé par les cellules de l'organe de l'émail. Celles-ci vont notamment induire l'arrêt de la prolifération des cellules ectomésenchymateuses proches, leur polarisation et la formation d'un prolongement cellulaire qui va s'allonger progressivement au fur et à mesure du dépôt de la dentine au pôle apical de la cellule. L'odontoblaste comprend ainsi, un long prolongement, totalement inclus dans la dentine, et un corps cellulaire localisé à l'interface pulpe-dentine (Couve et al., 2013). L'ensemble pulpe-dentine est appelé complexe pulpodentinaire. Parallèlement, les cellules ectomésenchymateuses centrales de la papille ectomésenchymateuse dentaire se différencient en fibroblastes dont la principale fonction est la synthèse et le renouvellement de la matrice extracellulaire pulpaire. Cette matrice est particulièrement riche en collagènes et en protéoglycanes. La transformation de la papille

ectomésenchymateuse en pulpe dentaire s'accompagne également d'un accroissement de la vascularisation du tissu, de sa colonisation par des cellules immunocompétentes chargées d'assurer sa surveillance, et de son innervation. La pulpe est ainsi un tissu conjonctif lâche fortement hydraté, richement innervé et vascularisé, et capable d'assurer sa défense, notamment en cas d'agression bactérienne.

1.3 Réponse de la pulpe dentaire à la lésion carieuse

Dans la cavité buccale, la dent est soumise à un environnement complexe dont certains éléments peuvent perturber l'équilibre physiologique de la pulpe. Par exemple, la dent peut être la cible d'attaques bactériennes qui vont déminéraliser et détruire progressivement l'émail et la dentine pour former une lésion carieuse : (Figure 6)**.**

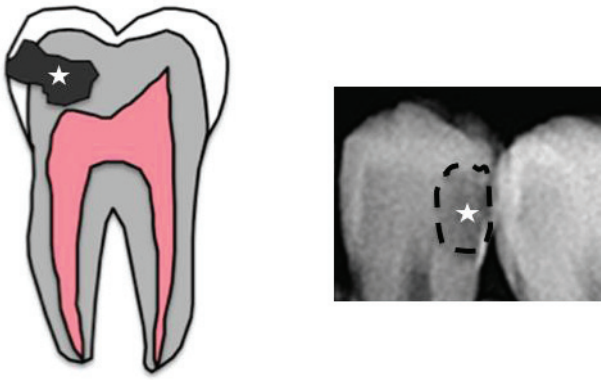

Figure 6 : Représentation schématique et radiographie d'une lésion carieuse profonde.

D'après le Larousse médical, la lésion carieuse est une « maladie détruisant les structures de la dent, évoluant de la périphérie (émail) vers le centre de la dent (pulpe dentaire) ». La carie dentaire serait la maladie qui affecte le plus d'êtres humains dans le monde : près de 2,4 milliards de personnes en 2010. Les soins dentaires liés à la carie auraient engendré la même année près de 450 milliards de dollars de dépenses (Figure 7)(Kassebaum et al. 2015)(Listl et al. 2015).

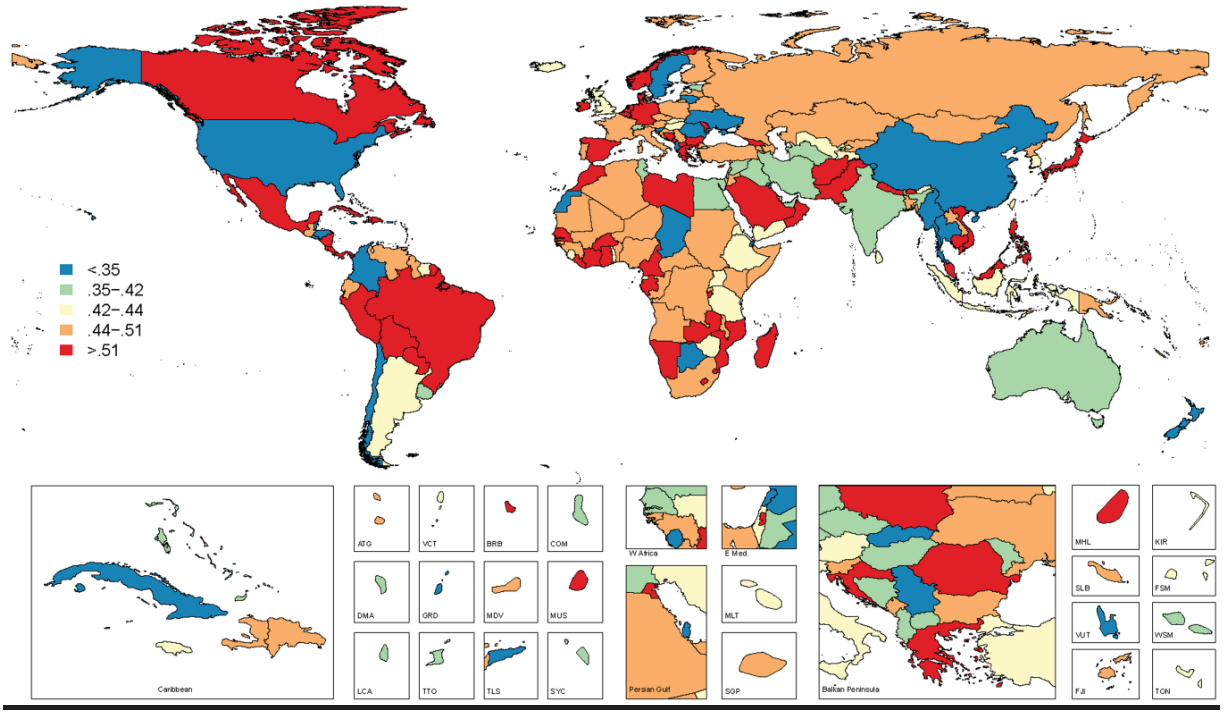

Figure 7 : Proportion de dents permanentes présentant une carie non traitée dans le monde en 2010. (Kassebaum et al. 2015)

Lorsqu'une lésion carieuse progresse à travers la dentine en direction de la pulpe, des mécanismes de défense et de réparation vont progressivement se mettre en place pour protéger la pulpe (Article 1).

Article 1 : Les mécanismes de défense et de réparation de la pulpe dentaire face à la carie

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Résumé : La carie dentaire est une maladie infectieuse chronique qui résulte de la pénétration de bactéries issues de la cavité buccale dans l'émail et la dentine. Les microorganismes déclenchent une réponse inflammatoire/immunitaire dans la pulpe dentaire qui peut permettre la cicatrisation du tissu pulpaire si l'infection n'est pas trop sévère et si l'émail et la dentine infectés sont intégralement retirés et la dent correctement restaurée. Cependant, une inflammation chronique persiste souvent dans la pulpe malgré le traitement, ce qui va entrainer une réduction de la capacité innée de la pulpe à cicatriser et à se réparer. Pour que la cicatrisation de la dent soit totale, une barrière de dentine réactionnelle/réparatrice doit être formée pour éloigner la pulpe des agents infectieux et des matériaux de restauration coronaire. Les données cliniques et les expérimentations *in vitro* indiquent clairement que la formation de cette barrière n'est possible que si l'inflammation et l'infection sont fortement atténuées et permettent le rétablissement de l'homéostasie tissulaire. C'est pourquoi favoriser la résolution de l'inflammation pulpaire pourrait constituer une solution thérapeutique valable pour assurer la pérennité des traitements dentaires. Cet article porte sur les mécanismes cellulaires et moléculaires clés impliqués dans la réponse pulpaire aux bactéries et dans la transition entre la réponse inflammatoire et la réparation dentinaire. Nous rapportons ici, à l'aide d'exemples choisis, plusieurs stratégies potentiellement utilisées par les odontoblastes et les cellules immunitaires spécialisées pour combattre les bactéries qui envahissent la dentine *in vivo*.

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Review Article

Dental Pulp Defence and Repair Mechanisms in Dental Caries

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Dental caries is a chronic infectious disease resulting from the penetration of oral bacteria into the enamel and dentin. Microorganisms subsequently trigger inflammatory responses in the dental pulp. These events can lead to pulp healing if the infection is not too severe following the removal of diseased enamel and dentin tissues and clinical restoration of the tooth. However, chronic inflammation often persists in the pulp despite treatment, inducing permanent loss of normal tissue and reducing innate repair capacities. For complete tooth healing the formation of a reactionary/reparative dentin barrier to distance and protect the pulp from infectious agents and restorative materials is required. Clinical and *in vitro* experimental data clearly indicate that dentin barrier formation only occurs when pulp inflammation and infection are minimised, thus enabling reestablishment of tissue homeostasis and health. Therefore, promoting the resolution of pulp inflammation may provide a valuable therapeutic opportunity to ensure the sustainability of dental treatments. This paper focusses on key cellular and molecular mechanisms involved in pulp responses to bacteria and in the pulpal transition between caries-induced inflammation and dentinogenic-based repair. We report, using selected examples, different strategies potentially used by odontoblasts and specialized immune cells to combat dentin-invading bacteria *in vivo*.

1. Odontoblasts in the Dental Pulp's Defence against Caries

The crowns of erupted human teeth are covered by symbiotic microbial communities, mainly composed of Gram-positive saprophytic bacteria which are normally harmless to the tooth. These communities adhere as biofilms to the highly mineralized enamel that constitutes a barrier which is impermeable to microorganisms and protects the underlying mineralized dentin and the loose connective tissue situated at the centre of the tooth, the dental pulp. However, when placed in a sugar-rich environment, specific bacterial populations from these communities release acids that progressively demineralize enamel [1, 2]. This leads to the appearance of a carious lesion characterized by a cavity within which "cariogenic" bacteria proliferate and release additional acids that progressively deepen the lesion. When the enamel barrier is disrupted, dentin becomes degraded by Gram-positive bacteria, including streptococci, lactobacilli, and actinomyces that largely dominate the dentin caries microflora [3]. The proliferation and metabolic activity of these microorganisms lead to the release of bacterial components into dentinal tubules and their diffusion towards the peripheral pulp. Dentin demineralization may also enable the release of bioactive molecules from the dentin matrix [4]. Recognition of bacterial components by host cells at the dentin-pulp interface triggers host protective events including antibacterial, immune, and inflammatory responses. These events may

eliminate early stage bacterial infection and block the route of its progression when accompanied by dentin formation at the pulp-dentin interface. Unchecked, bacterial invasion results in irreversible chronic pulp inflammation, most often after a long phase of chronic inflammation. Subsequently, pulp necrosis, infection of the root canal system, and periapical disease may occur [3, 5]. Pulp inflammation, also called "pulpitis," generally dampens after microorganism removal by the dental practitioner and neutralization of intratubular diffusing components by the pulp immune system, both decreasing the production of proinflammatory mediators [6]. However, when the caries lesion is close to the dentin-pulp interface, pulpal inflammation does not resolve completely after dental treatment and may become low-grade and chronic in nature. This chronic inflammation is responsible, as in other connective tissues, for the permanent loss of normal tissue function and the reduction of defence capacities to future injuries. On occasions, rapid cessation of inflammation enables complete pulp healing with the formation of a barrier of reactionary dentin by the original surviving odontoblasts and/or reparative dentin by newly differentiated odontoblast-like cells in animal models [7]. Dentin neoformation protects the underlying pulp from the dentin infection and the crown filling biomaterial, thus reducing the risk of permanent irritation by external bacterial or chemical agents. It is reasonable to speculate that rapid reactionary/reparative dentin formation is initiated, the quicker pulp healing occurs, and health is reestablished. So, from a clinical point of view, it appears crucial to identify molecular and cellular agents able to dampen immune/inflammatory events within the dental pulp and promote rapid return to tissue homeostasis and health once the bacterial infection is resolved [2, 8–10]. Such agents should help to prevent the evolution of the pulp inflammation towards becoming chronic in nature. To identify these agents, it is important to gain an in-depth knowledge of the events that initiate and control the early steps of human pulp antibacterial defence and dentinogenesis-based reparative mechanisms in caries-affected human teeth. This paper focusses on key cellular and molecular mechanisms involved in pulp responses to bacteria and in the pulpal transition between caries-induced inflammation and dentinogenic-based repair. We report, using selected examples, different strategies potentially used by odontoblasts and specialized immune cells to combat dentin-invading bacteria *in vivo*.

Odontoblasts are the first pulpal cells encountered by dentin-invading pathogens and their released products owing to both their specific localization at the pulp-dentin interface and the embedding of their long cellular processes in dentin tubules. We and others have therefore hypothesized that, in the tooth, they represent the first biologically active line of defence for the host, fulfilling the role devoted elsewhere in the body to skin and mucosal epithelial cells [12, 13]. Odontoblasts may thus be involved in combatting bacterial invasion and activating innate and adaptive aspects of dental pulp immunity. Both these events can only be activated following pathogen recognition by pulp cells. In a general way, such recognition occurs through the detection ("sensing") of molecular structures shared by pathogens and that are essential for microorganism survival. These structures are termed Pathogen-Associated Molecular Patterns (PAMPs) and are sensed by a limited number of so-called Pattern-Recognition Receptors (PRRs). One important class of PRRs is represented by the Toll-like receptor (TLR) family that is crucial for the triggering of the effector phase of the innate immune response [14–16]. TLR2 and TLR4, which are involved in Gram-positive and Gram-negative bacterial sensing, respectively, have been previously detected in the odontoblast cell membrane in healthy pulp, indicating that odontoblasts are equipped to recognize these pathogens when they diffuse through dentin tubules during the carious infection [13, 17]. TLR2 has been shown to be upregulated in odontoblasts beneath caries lesions compared with odontoblasts beneath healthy dentin [2], suggesting that these cells are not only adapted to the recognition of Gram-positive bacteria but that they are also able to amplify their response to these pathogens.

One major consequence of TLR activation is upregulation of innate immunity effectors, including antimicrobial agents and proinflammatory cytokines and chemokines that recruit and activate tissue resident and blood borne immune/inflammatory cells [18, 19]. Odontoblasts have been found to produce several antibacterial agents, among which beta-defensins and nitric oxide have received particular attention. Beta-defensins (BDs) are cationic, broad-spectrum antimicrobial peptides that kill microorganisms by forming channel-like micropores that disrupt membrane integrity and induce leakage of the cell content [20–23]. They are mainly produced by epithelial and immune cells to protect skin and internal mucosae from pathogen invasion. Whereas BD-1 is generally constitutively expressed, BD-2, BD-3, and BD-4 are induced by microorganisms that come into contact with host cells. Several *in vitro* studies have reported that BDs might also be involved in the pulpal defence against caries-related microorganisms. Indeed, BD-2 was shown to possess antibacterial activity against *S. mutans* and *L. casei* [24–26] and BD-3 exhibited antibacterial activity against mature biofilms containing *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus mutans*, and *Enterococcus faecalis* [27]. A proinflammatory role was also proposed for BD-2, which upregulates interleukin (IL-) 6 and as Chemokine [C-X-C Motif] Ligand 8 (CXCL8, also known as IL-8) in odontoblast-like cells *in vitro* [28]. A positive feedback mechanism could exist between inflammatory cytokines and BD-2, the expression of which was found to be stimulated by IL-1α and tumor necrosis factor (TNF-) α in cultured human dental pulp cells [29, 30]. The proinflammatory effect of BD-2 could be augmented by the fact that it chemoattracts immature antigen-presenting dendritic cells (DCs), macrophages, CD4+ memory T cells, and natural killer (NK) cells by binding to cell surface chemokine receptors [22]. *In vitro*, odontoblast BD-2 gene expression was not modified by TLR2 activation in a tooth organ culture model, whereas BD-1 and BD-3 genes were downregulated [13]. BD-2 gene expression was upregulated upon TLR4 activation, which suggests that BDs are differentially produced by odontoblasts to combat Gram-positive and Gram-negative bacteria. *In vivo* studies have revealed that odontoblasts in healthy pulp synthesize

BD-1 and, to a lesser extent, BD-2 [31, 32]. Constitutive expression of low levels of BDs in the odontoblast layer might be necessary to destroy individual or very small groups of oral early stage bacterial invaders which enter the tooth through tiny, clinically undetectable lesions such as enamel cracks, before these bacteria engage with the pulpal immune system. Discrepancies exist between reports regarding the regulation of BDs in inflamed dental pulp. Indeed, BD-1 and BD-2 were first reported to be decreased during irreversible pulpitis [28], whereas, in a more recent study, BD-1 and BD-4 were found to be increased in inflamed pulps compared with healthy ones; the expression of BD-2 and BD-3 however remained constant [32]. Differences in the inflammatory status between pulp samples (reversible versus irreversible inflammation) may be responsible for these discrepancies. It remains unclear as to whether BDs are present in the bacteria-challenged inflamed pulp at levels that enable them to play a major role in the tissue defence against dentin-invading bacteria. Further studies are needed to investigate the antibacterial activity of BDs produced at *in vivo* relevant concentrations by odontoblasts challenged with caries-related microorganisms. Another important antimicrobial agent produced by odontoblasts challenged with microbial components is nitric oxide (NO). NO is a potent antibacterial, highly diffusible free radical produced from L-arginine through oxidation by NO synthases (NOS), of which there are 3 isoforms: NOS1 (neuronal NOS) and NOS3 (endothelial NOS), that are constitutively expressed in most healthy tissues, and NOS2 (inducible NOS), generally absent from healthy tissues and induced in particular in tissues challenged by microorganisms. NOS1 and NOS3 are constitutively expressed in physiological conditions by many cells and produce very low, picomolar to nanomolar range NO concentrations within seconds or minutes. NOS2 is mostly involved in host defence by producing high, micromolar range amounts of NO for sustained periods of time (hours to days) [33–39]. NOS2 is not, or only moderately, expressed in healthy human dental pulps and was found to be rapidly upregulated in inflamed pulps [40–44]. Furthermore, NOS2 activation was shown to promote the accumulation of neutrophils and macrophages in experimentally inflamed rat incisor pulps [42, 43]. CXCL8 might also be involved in this process since NO has been shown to stimulate the production of this chemokine in human pulp cells *in vitro* [45]. Human odontoblasts in the inflamed dental pulp showed a marked immunoreactivity for 3-nitrotyrosine (a biomarker for NO-derived peroxynitrite), suggesting that these cells release NO upon NOS2 activation [44]. Indeed, NO release might constitute an important defence mechanism against *Streptococcus mutans* as the growth of these microorganisms has been shown to be inhibited by NO *in vitro* [46]. Accordingly, NO produced at high concentration by NOS2 in the inflamed pulp might be used by odontoblasts as a weapon to combat cariogenic bacteria. We have recently presented evidence that odontoblasts differentiated *in vitro* strongly amplify their NOS2 synthesis and NO production upon TLR2 activation. The NO produced was found to inhibit the growth of *Streptococcus mutans*, thus suggesting the role of this odontoblast-derived molecule in the limitation of the intradentinal progression of caries-related microorganisms [47].

Numerous *in vitro* studies have also shown that odontoblasts produce inflammatory cytokines and chemokines when challenged by PAMPs from Gram-positive bacteria [12, 13]. In particular, odontoblasts differentiated *in vitro* were found to be responsive to lipoteichoic acid (LTA), a Gram-positive bacteria wall component recognized at the cell surface through TLR2. Engagement of odontoblast TLR2 by LTA upregulated TLR2 itself and NOD2, a cytosolic PRR, which led to nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) signalling activation, dentinogenesis inhibition, and production of the proinflammatory chemokines Chemokine [C-C Motif] Ligand 2 (CCL2), CXCL1, CXCL2, CXCL8, and CXCL10 [2, 12, 48–51]. Chemokine production by odontoblasts following bacterial challenge might attract immune cells into the odontoblast layer beneath the carious lesion [52]. Indeed, when dentin is being demineralised by caries, immature DCs accumulate at an early stage at the dentin-pulp interface in a strategic location to capture foreign antigens. A progressive and sequential accumulation of T cells (= T lymphocytes), macrophages, neutrophils, and B cells (= B lymphocytes) then occurs in the pulp, concomitantly with the deepening of the dentin lesion, the increase of the bacterial insult, and the development of the pulp inflammatory process [6, 53]. Thus, it is likely that odontoblasts are able to attract some, if not all, of these immune cell populations at the pulp-dentin interface to neutralize bacterial by-products that reach the pulpal end of the dentin tubules. By using culture supernatants of odontoblast-like cells stimulated with TLR2 agonists, we demonstrated that odontoblasts produced chemokines able to recruit immature DCs [12, 48]. CCL2, strongly expressed in odontoblasts beneath dentin carious lesions, may be involved in this process since it is a key element in the recruitment of circulating blood dendritic cells. Odontoblast-derived CXCL1, CXCL2, and CXCL8, which are known to attract neutrophils, and CXCL10, known to attract T cells, could be involved in the accumulation of other populations of immune cells at the dentin-pulp interface. However, to our knowledge, no direct evidence for a role of odontoblast-derived chemokines in these processes has been reported so far.

IL-6 is a pleiotropic cytokine produced by a variety of immune and nonimmune cells that regulates many aspects of the local immune response [54]. It is strongly upregulated in bacteria-challenged inflamed pulps *in vivo* and in odontoblasts *in vitro* upon TLR2 engagement [49, 55]. IL-6 is notably critical to the differentiation and regulation of T helper (Th)2, Th17, and T regulatory (Treg) phenotypes, and it promotes the secretion of acute-phase proteins including lipopolysaccharide-binding protein [19]. All these functions might be undertaken in inflamed pulps by IL-6. Since it also increases vascular permeability, IL-6 might also be involved in the formation of oedema induced by the progressive intradentinal penetration of Gram-positive oral bacteria [49].

IL-10 is an immunosuppressive cytokine produced by many immune and nonimmune cells which modulate immune responses to microbial antigens in order to prevent excessive or unnecessary inflammation. It acts in particular by decreasing the production of the proinflammatory

cytokines IL-6 and CXCL8, thereby suppressing inflammation-associated immune responses and limiting damage to the host [56]. It also inhibits Th1 and Th2 immune responses but promotes the differentiation of regulatory T cells which control excessive immune responses in part by producing IL-10, which provides a positive regulatory loop for IL-10 induction [57, 58]. We found that IL-10 is upregulated in bacteria-challenged inflamed pulps *in vivo* [49] where it might help limit the spread of pulp inflammation which is initially restricted to the dentin-pulp interface beneath early dentin caries lesions [59]. IL-10 was upregulated in odontoblast-like cells *in vitro* upon TLR2 engagement, suggesting that odontoblasts are capable not only of initiating the pulp immune and inflammatory response to dentin-invading bacteria, but also of limiting its intensity [49].

Recently, we have studied the role of lipopolysaccharide-binding protein (LBP), an acute-phase protein known to attenuate proinflammatory cytokine production by activated macrophages. LBP has been shown to prevent the binding to host cells of several bacterial cell wall components including lipopolysaccharides, lipoteichoic acids, lipopeptides, and peptidoglycan [60]. It was also found to transfer lipopolysaccharides to high-density lipoproteins in the plasma for neutralization [61]. We recently detected LBP synthesis and accumulation in bacteria-challenged inflamed pulp, whereas this protein was not found in healthy pulp. *In vitro*, LBP was upregulated by Pam2CSK4 (a diacylated lipopeptide synthetic analog that binds specifically TLR2) in odontoblasts differentiated *in vitro*. It also decreased TLR2 activation and attenuated proinflammatory cytokine synthesis ([62], unpublished results). This molecule might be involved in the neutralization of bacterial components that gain access to the pulp, thus limiting activation of the pulp immune cells and the associated inflammatory response to dentin-invading bacteria [8].

In summary, numerous studies performed over the last decade have shown that odontoblasts are able to detect oral microorganisms that invade mineralized dental tissues from the oral cavity. They mobilize themselves against this threat by building their own antibacterial arsenal (defensins, nitric oxide) and by sending molecular messengers (chemokines, cytokines) to the neighbouring pulp to alert immune cells able to mount responses to microorganisms (Figure 1). However, the majority of these studies have been performed *in vitro* and currently minimal information is available about the nature and role of antibacterial and immune effectors in caries-affected teeth *in vivo*. Additional experiments are therefore warranted to further characterize the molecular effectors and regulators of human dental pulp immunity and determine their therapeutic potential to promote the recovery of dental pulp homeostasis and health.

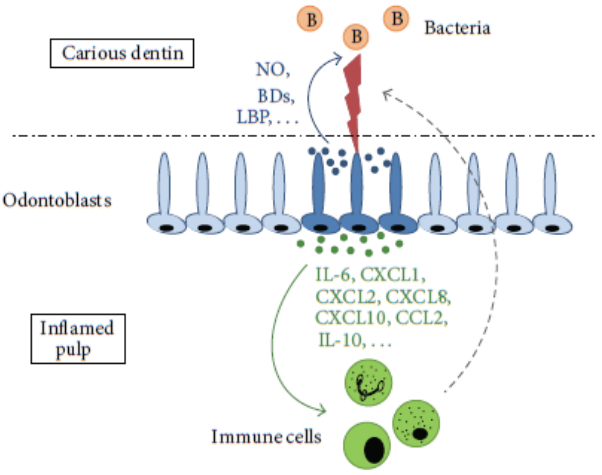


FIGURE 1: Two key aspects of the odontoblast defence against dentin-invading bacteria. Bacteria (B) present in the carious dentinal lesion release pathogenic components that activate (blue arrow) odontoblasts (dark blue) adjacent to the lesion, triggering the production of antibacterial molecules (blue dots). These molecules diffuse through dentin tubules in an attempt to destroy the invading microorganisms (NO, BDs) or considerably decrease their pathogenicity (LBP). In parallel, proinflammatory and immunomodulatory mediators (green dots), including IL-6, IL-10, CXCL1, CXCL2, CXCL8 (IL-8), CXCL10, and CCL2, are secreted by odontoblasts at the opposite cell pole and diffuse into the subodontoblast pulp area (green arrow) where they activate and mobilize various populations of immune cells (as described in the main text body) enabling the immunosurveillance of the tissue. Immune cells then migrate (dotted grey arrow) towards the pulp-dentin interface beneath the lesion to combat the bacteria and coordinate the immune defense response.

2. Response of Pulp Immune Cells to Tooth-Invading Pathogens

As stated above, eliminating the decayed mineralized tissues containing microbial agents can result in decreased pulpal inflammation, promotion of tissue healing, and restoration of the normal biological functions of the pulp. Like peripheral organs and tissues such as skin, gastrointestinal tract, and lungs, healthy dental pulp contains sentinel leukocytes, which are able to biologically sample and respond to the local environment, including macrophages, DCs, and T cells [52, 53, 63, 64]. Fluorescence-activated cell sorting (FACS) analysis of enzymatically digested whole pulp tissue revealed that leukocytes represent ~1% of the total cell population in nonerupted human third molars [10]. Leukocytes in healthy tissue undertake immunosurveillance, that is, continuous sampling of their environment to sense microorganisms invading into the body. Their numbers significantly increase when pathogens are detected, due to the elevation of the inflammatory process. This inflammation is part of the normal protective immune response of the host to tissue infection and during this response, leukocytes from the circulatory system are triggered to adhere to endothelial cells lining blood vessels prior to them migrating out of the blood vessel to the site of infection. Neutrophils are initially recruited to the inflamed tissue to engulf and destroy invading microorganisms; subsequently this response is followed by monocytes which also differentiate into macrophages. In teeth, neutrophils and macrophages progressively infiltrate the pulp tissue as the carious disease progresses [4, 6, 9, 53, 65–67].

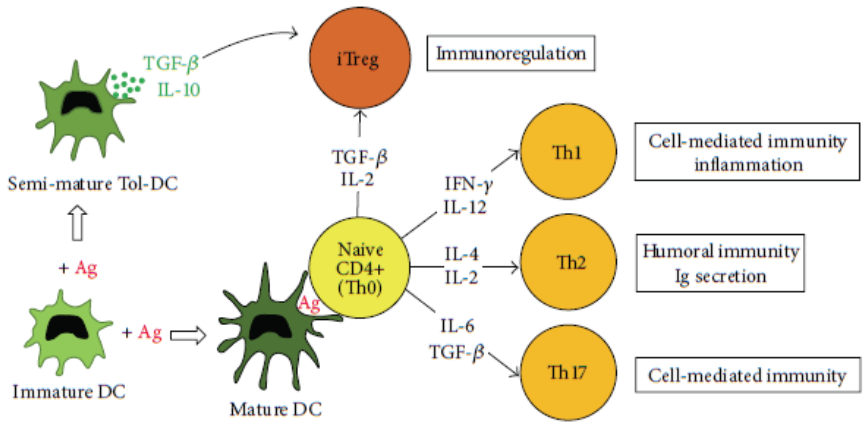


FIGURE 2: The putative role of dendritic cells (DCs) in the regulation of T helper (Th) and induced regulatory T (iTreg) cell differentiation. Upon encountering antigens (Ag), immature DCs usually become mature DCs which present antigens to naive CD4+ (Th0) cells. Upon antigen recognition, Th0 cells clonally expand and can differentiate into various subsets of effector cells (Th1, Th2, or Th17) or into iTreg cells depending on the cytokines present in their environment. Alternatively, immature DCs can mature only partially to become Tolerogenic-DCs (Tol-DCs) which can directly induce iTreg cell differentiation through TGF-β and IL-10 secretion. IL, interleukin; IFN, interferon; TGF, transforming growth factor; Ig, immunoglobulin.

Macrophages are able to phagocytose bacteria and activate T cells triggering an adaptive immune response which occurs in association with DCs. In the pulp, DCs are initially present in an immature state and are attracted by odontoblast-derived chemokines to the site of infection, where they capture bacterial antigens diffusing through dentin tubules towards the pulp [6, 12, 48, 53]. Antigen uptake triggers the activation and progressive maturation of DCs, and they subsequently migrate to regional lymph nodes where they present antigens to, and activate, naive CD4+ T cells (also called Th0 cells). Activated DCs secrete a range of cytokines that influence both innate and adaptive immune responses, and they are considered key regulators of the tissue's defence against infection. Naive CD4+ T cells, when activated, can differentiate into effector CD4+ T helper cells or induced regulatory T (iTreg) cells [68]. Furthermore effector CD4+ T cells are classically assigned to Th1, Th2, or Th17 subsets and undertake specific functions in the immune response including regulation of cell-mediated immunity, inflammation, and protection against intracellular pathogens. Th1 cells are generated by IL-12 and interferon (IFN-) γ exposure and they secrete IFN-γ, IL-2, and TNF-α. Naive CD4+ T cells differentiate into Th2 cells following exposure to IL-4 and IL-2. Th2 cells produce IL-4, IL-5, IL-6, IL-10, IL-13, and IL-14; they regulate humoral (immunoglobulin-mediated) immunity and are involved in protection against extracellular pathogens. The Th17 lineage pathway provides a unique mechanism for protection against bacterial and fungal pathogens through the production and induction of inflammatory cytokines and the recruitment of neutrophils. Th17 cells are induced to differentiate from naïve CD4+ T cells mainly by transforming growth factor (TGF-) β and IL-6 [69] (Figure 2). We have previously provided precise quantification of T cells in healthy human dental pulp, enabling a better understanding of the initial capacity of the pulp to detect and combat pathogens. Our data demonstrated that cytotoxic CD8+ T cells represented ~21% total leukocytes, and CD4+ T cells were ~11%, with DCs ~4% of the leukocyte population. We observed that progressive and sequential accumulation of CD4+ and CD8+ T cells was observed in inflamed pulp which occurred in parallel with the deepening of the dentin lesion [4, 53, 67]. Elucidating the exact mechanisms that regulate Th1, Th2, or Th17 responses is essential to more comprehensively understand pulp pathogenesis; however to date no data are available regarding the subsets of T cells involved in these mechanisms. Thus far only one study has reported pulp regeneration in a mild irreversible pulpitis model after inhibition of IL-6 secretion by matrix metalloproteinase (MMP-) 3. The authors proposed that the control of IL-6 activities by MMP-3 could thus decrease the Th2 response and Th17 cell induction [70]. NK cells are also a well-known arm of the innate immune system. They are reported to exhibit features characteristic of the adaptive immune response and they have recently been identified in healthy rat molar pulps [71]. We have now found that NK cells represented ~2.5% of leukocytes in human healthy pulp [10]. In addition, a subset of T cells known as natural killer T (NKT) cells has been detected in healthy rat pulp [71] and these cells are known to play a major role in the development of Th1 versus Th2 immune responses [72]. Finally, a relatively small number of B cells are present in healthy pulp tissue and their numbers significantly increase during pulpitis and caries progression [10, 73]. Immunohistochemical analysis of inflamed pulp demonstrated that B cell-derived IgG1, rather than IgG2, is the dominant subclass of immunoglobulin followed by IgA and IgE [4, 65]. During human dental root resorption, B cells form clusters in the pulp of deciduous teeth [74] and their role may be to modulate DC functions [75].

In order to avoid irreversible damage to the pulp tissue, the complex immune responses must be controlled to enable

pathogen destruction without causing damage to the host. Regulatory cells play a major role in this process [76]. In particular, subpopulations of immature DCs, called Tol-DCs, are resistant to maturation and are implicated in the regulation of the immune response [77]. They induce central and peripheral tolerance through different mechanisms including T cell depletion or anergy, induced Treg cell differentiation from naive CD4+ T cells, and production of a variety of immunomodulatory mediators such as PD-L1, PD-L2, heme oxygenase-1 (HO-1), HLA-G, galectin-1, DC-SIGN, IL-10, TGF-β, indoleamine 2,3-dioxygenase, IL-27, and NO [78, 79]. Naive CD4+ T cells differentiate into induced Treg cells (iTregs) following exposure to TGF-β and IL-2. They express CD4, CD25, and FoxP3 and secrete TGF-β and IL-35 that inhibit the effector T cell response. Among the iTreg population, Tr1 cells secrete a large quantity of IL-10 and TGF-β which suppress Th responses [80]. Relatively large numbers of iTregs have been detected in intensely inflamed human pulps [81]. FACS analysis, using healthy human molars, resulted in the detection of iTregs identified by the phenotype CD45+CD3+CD4+CD127low CD25+ and Foxp3+. There is also now evidence for the presence of a specific subset of DCs expressing HO-1 in healthy human pulp [10]. DCs expressing HO-1 have immunoregulatory properties, as this enzyme protects cells against inflammatory and oxidative stress [82]. Furthermore, myeloid derived suppressor cells (MDSCs) have been identified in healthy pulp and they constitute a heterogeneous population of cells with a remarkable ability to regulate immune responses [83–85]. Notably MDSCs expanded by exposure to bacterial components, such as lipopolysaccharide (LPS), regulate alloreactive T cells via HO-1 and IL-10 secretion [86]. Together, these results indicate that healthy dental pulp is equipped for limiting or fine-tuning innate and adaptive responses even in the absence of pathogens.

In summary, healthy dental pulp contains resident immune cells and is thus initially well equipped to detect and mount effective immune responses against invading pathogens. Recruitment of circulating immune cells into the pulp tissue during the inflammatory process reinforces its defence potential. In particular, it has recently been reported that the range of resident leukocytes is much wider in healthy pulp than previously understood and includes several populations of cells with immunoregulatory properties. These data indicate that the immune and inflammatory dental pulp response to pathogens is extremely complex. Additional studies are therefore warranted to understand how such a response can be controlled to promote tissue healing after pathogen removal by the dental practitioner.

3. Inflammation-Regeneration Interplay in the Dentin-Pulp Complex

Clearly, defence and reparative responses within the tooth are inextricably linked. During carious disease, which damages the tooth structure, the host aims to both fight the infection, via its immune-inflammatory response, and "wall off" and restore the tooth structure, via its dentinogenic responses.

Notably, the regenerative mechanisms within the dental tissues are underpinned and informed by developmental processes. Following a series of molecular and cellular signalling events which occur between the developmental epithelium and mesenchymal tissue, odontoblasts differentiate from progenitor cells bordering the dental papilla. In brief, they take on a polarised columnar form and secrete predentin and further signalling leads to cells of the inner enamel epithelium, which are in contact with the predentin, differentiating into polarised columnar ameloblasts, which subsequently synthesise the enamel. The predentin is converted to dentin and further cycles of predentin secretion and mineralisation result in the odontoblasts receding from the dentinoenamel junction towards the pulp core. As the dentin structure of the tooth develops, the odontoblasts leave their cellular processes extended within the dentinal tubules. A multitude of genes have been identified as being active during tooth development and morphogenesis, which indicates the complexity of the process [87]. Indeed, many of the growth factors involved in signaling the dentinogenic process subsequently become fossilised within the dentin as they are secreted by the odontoblast during development. Notably, their later release from the dentin during disease is understood to regulate both regenerative and defensive responses within the tooth and is discussed in more detail below.

Whilst primary dentinogenesis occurs at a rate of ~4 μm/day of dentin deposition during tooth development, secondary dentinogenesis decreases to a rate of ~0.4 μm/day following root formation and continues to occur throughout the life of the tooth. Tertiary dentinogenesis however describes the process of hard tissue repair and regeneration in the dentin-pulp complex, which is the tooth's natural wound healing response. With milder dental injury, such as early stage dental caries, primary odontoblasts become reinvigorated to secrete a reactionary dentin which is tubular and continuous with the primary and secondary dentin structures. However, in response to injury of a greater intensity, such as a rapidly progressing carious lesion, the primary odontoblasts die beneath the lesion [88, 89]. While it is not entirely clear what causes this odontoblast cell death, it is hypothesized that bacterial toxins, components released from the demineralised dentin or even local generation of high levels of proinflammatory mediators, signal this event. Subsequently, however, if conditions become conducive (e.g., if the carious infection is controlled or arrested), stem/progenitor cells within the pulp are signalled to home to the site of injury and to differentiate into odontoblast-like cells. These cells deposit a tertiary reparative dentin matrix, reportedly at a similar rate to that of primary dentinogenesis, and this clinically results in dentin bridge formation. The new hard tissue deposited walls off the dental injury and the infecting bacteria, protecting the underlying soft tissues, and partially restores tooth structure [90]. Clearly the relative complexity of these two tertiary dentinogenic processes differs, with reactionary dentinogenesis being comparatively simple and requiring only upregulation of existing odontoblast activity, whereas reparative dentinogenesis is more complex and involves recruitment, differentiation, and upregulation of dentin synthetic and secretory activity. Notably, it is

understood that tertiary dentin deposition rates somewhat recapitulate those in development with dentin. Tertiary dentinogenic events are also understood to be signalled by bioactive molecules, similar to those present during tooth development. Some of these molecules may arise from the dentin when it is demineralised by bacterial acids as a variety of growth factors and other signalling molecules are sequestrated within the dentin during its deposition and formation [90–92]. The breakdown and release of signalling molecules from the dentin provide a means by which the tooth can detect tissue damage and subsequently rapidly respond. Indeed, an array of molecules are bound within dentin and are known to be released from their inactive state by carious bacterial acids, as well as restorative materials, such as calcium hydroxide, which are known to stimulate dentin bridge formation following clinical application. Furthermore a variety of molecules which in general are regarded as inflammatory mediators are also implicated in signalling repair responses. Clearly, it is likely that a fine balance exists between their levels and temporal and contextual profiles, which subsequently regulates the effects of these molecules on dental cells and tissues. These signalling aspects are further discussed below in more detail.

The carious infection, if unchecked, will progress through the dental hard tissues and into the soft pulpal core. In general, markers of the inflammation also subsequently increase including levels of cytokines and the immune cell infiltrate [64, 73, 93]. Indeed, the increased levels of cytokines have a range of regulatory functions including lymphocyte recruitment, extravasation, activation, differentiation, and antibody production. The roles of the cytokines, IL-1α, IL1-β, and TNF-α, are particularly well characterized in orchestrating the immune response in the pulp in response to carious and deeper associated periapical infections [93–100]. Initially, as has been discussed, resident pulp cells, including odontoblasts, will increase their expression of these molecules; however, a range of immune cells recruited to the lesion in response to infection will further add to the molecular milieu. Furthermore, components of dentin released by carious bacterial acids during the demineralization process have also been demonstrated to contribute to the levels of inflammatory mediators [101]. Notably, many other cytokines including IL-4, IL-6, IL-8, and IL-10 have been shown to be increased in pulp tissue, which is affected by carious disease [102–104]. It is a range of these potent cytokine signaling molecules which generates the chemotactic gradients leading to recruitment and activation of the immune cells described above and can subsequently lead to the chronic cycle of inflammation present within the tooth [105, 106].

Notably, the cytokine IL-8 is constitutively expressed by odontoblasts, likely in anticipation of disease events, and its levels can be significantly upregulated both by bacterial components (e.g., LPS via TLR signaling mechanisms) and by IL-1β and TNF-α in a range of cell types [107]. IL-8 is particularly important in the recruitment and activation of neutrophils, which are generally one of the first immune cell types present at the site of infectious disease (as described in detail above). Interestingly, we have reported elevated levels at both the transcript and protein levels for a range of proinflammatory mediators, including S100 proteins, in carious diseased pulpal tissue compared with healthy pulpal tissue [66, 93].

While local release and accumulation of proinflammatory mediators occur in response to the progressing carious infection, data now indicate that bacterial acid-driven dentin demineralization likely adds to the complex cocktail of signaling molecules present within the diseased dental tissue [66]. As we are aware that odontoblasts basally express certain cytokines [107], it is therefore perhaps of little surprise that these bioactive molecules become sequestrated within the dentin for later release when it is demineralised during the disease process. Indeed, the components of the dentin matrix are clearly multifunctional and can stimulate multiple processes such as promoting mineralization and stimulating cell migration and activation [92, 100, 101, 108].

The extravasation and antimicrobial activity of immune cells within the pulp result in the release of molecules that, while aimed at combatting the bacterial infection, can however also cause significant collateral host tissue damage. Degradative enzymes, such as MMPs necessary for the immune cell migration through the soft tissue matrix, cause degradative damage and the increased levels of reactive oxygen species (ROS) utilized by immune cells for antimicrobial action also damage host cells and tissues. These events can contribute to the chronic cycle of inflammation as these molecules are also known to have direct proinflammatory actions. Indeed, ROS, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, can stimulate cytokine release by activating the key proinflammatory intracellular signaling pathways regulated by the p38 MAPK and NF-κB proteins in several immune and tissue structural cell types [13, 109, 110]. Notably, these pathways have become exceedingly well characterized in the proinflammatory process and are central to extracellular signal transduction in response to cellular stresses, such as infection and cytokine stimulation [111, 112]. It should however be noted that while the activation of these signaling pathways is generally regarded as being involved in the amplification of the immune and inflammatory responses, they also appear to associate with repair and regeneration signaling. Indeed, while generally it is regarded that tissue repair does not occur until infection is under control and the inflammation is modulated, the magnitude and temporospatial nature of events may be key to fine-tuning this complex response. The link between inflammation and regeneration via these intracellular signaling interactions will be further discussed below.

Notably, the dentin-pulp complex has significant regenerative potential following injury due to its tertiary dentinogenic responses. Due to the differences in complexity of the cellular processes involved in reactionary or reparative dentinogenesis, the local inflammatory response will likely have differing effects at the different stages within it [66]. It is notable that tissue reparative events will likely only occur when the infection and inflammation are under control and this may result from the immune response resolving the infection, or following clinical intervention to remove the disease. This balance between defence and repair in the tissue is clearly important. Indeed, it would not appear practical for

body resource to be utilized to rebuild tissue, which remains under attack from infection and hence may continue to break down. Furthermore, from a clinical standpoint, if the tissue is rebuilt while the infection is still present, this may prove futile and likely result in the need for retreatment.

In support of this premise, several lines of evidence indicate that chronic pulpal inflammation impedes reparative processes and the accepted paradigm is that regeneration only follows after appropriate resolution of inflammation, which likely occurs after disinfection [113–115]. Indeed, we know that while the immune-inflammatory responses aim to be protective, tissue damage occurs collaterally due to the release of degradative molecules and enzymes, as described above, and hence any reparative mechanisms ongoing may not be apparent. Potentially, the most significant evidence that resolution of infection and inflammation are necessary to enable regeneration is derived from classical animal studies, which demonstrated that repair was apparent only in artificial cavities made in germ-free animals compared with those where the cavities were infected and subsequent inflammation occurred [116]. Further evidence regarding the effects of inflammation on regeneration comes from *in vitro* studies that demonstrate the biphasic responses of pulp cells to proinflammatory signaling molecules. Notably, while relatively low levels of cytokines and growth factors can be stimulatory to cells, high levels of these molecules, such as TNF-α and TGF-β, present during infection and inflammation can cause cell death [97, 108, 117, 118]. More direct evidence also comes from studies that demonstrate stem cell differentiation processes are clearly impeded by proinflammatory signaling [119, 120].

Recent work has, however, indicated that inflammatory signals can stimulate repair processes (reviewed in [121]). Indeed, signal transduction via both the key proinflammatory MAPK and NF-κB pathways (as described above) is also implicated in several reparative response processes. Data from several sources have demonstrated that these intracellular cascades can be activated in dental cells by several inflammation-related molecules, including bacterial components, ROS, and cytokines, which subsequently drive *in vitro* mineralization and differentiation responses. Arguably, it may be that acute or low levels of these inflammatory signals are necessary to signal these regenerative responses [109, 122–128]. Interestingly, it is also known that dying cells release and promote local secretion of low levels of proinflammatory mediators as damage-related signals [129]. Potentially, this sterile inflammation may occur during pulpal fibroblast senescence in the aging pulp and, subsequently, this process may generate nucleation points which drive pulp stone formation [130]. Combined, these data indicate that a delicate balance exists between the signaling or inhibition of repair and regeneration by proinflammatory mediators. Subsequently, we hypothesize that relative low level or acute inflammation may stimulate tissue regeneration, whilst higher chronic levels may impede the reparative processes and favor intense immune cell recruitment and activation.

Intriguing evidence linking the two processes of repair and regeneration can also be derived from data which demonstrates the sharing of receptors between immune and repair-related cells. Indeed, the C-X-C chemokine receptor 4 (CXCR4) is known to be expressed on both of these two different cell types [131, 132]. Furthermore, both the receptor and its ligand, stromal cell-derived factor-1 (SDF-1)/CXCL12, have been detected within the dentin-pulp complex and are reportedly upregulated during dental disease [133, 134]. Potentially, the sharing of this chemotactic receptor by these cell types appears somewhat logical as tissues which are damaged or infected, as is the case with the tooth during caries infection, need to recruit both immune and stem cells to injury sites to facilitate defence and repair [135]. The regulation as to which of these two processes predominates may, however, be locally controlled as studies have shown that cytokine levels modulate the stem cell surface expression of CXCR4. It is therefore conceivable that relatively high levels of proinflammatory molecules may abrogate CXCR4-mediated stem cell response at sites where inflammation is overriding [131].

Further support for the role of inflammation events preceding repair is potentially provided clinically following the application of the chemically related pulp capping agents of calcium hydroxide and Mineral Trioxide Aggregate (MTA). These restorative agents are known to enable the formation of tertiary dentin, in the form of a dentin bridge, beneath the site of application. Notably, however, chronologically prior to visible signs of hard tissue healing process, dental tissue inflammation is routinely observed histologically [136]. While calcium hydroxide has been applied clinically for over 60 years [137–140], its mechanism of action in the induction of reparative dentinogenesis remains controversial, although its beneficial effects have been attributed to the local release of hydroxyl ions [139], which raise pH and lead to cellular necrosis [141, 142]. Hence, it is the nonspecific chemical tissue irritation effect of these restoratives which has been cited as their principal mechanism of action for promoting dentin-pulp complex tissue regeneration. More recent studies have also indicated that these regenerative effects are perhaps more related to their ability to sterilize the site of infection whilst releasing bioactive signaling components from the dentin [143, 144]. It could therefore be hypothesized that a combination of events may occur to facilitate dentin-pulp complex repair *in vivo* following their placement. Indeed, the local cellular necrosis may stimulate sterile inflammation [145–148], which is able to resolve due to the elimination of bacteria by the combination of the material and clinical procedure. This relatively mild and acute immune response combined with the leaching of growth factors and signaling molecules from the dentin may subsequently generate a conducive environment for reparative dentinogenesis [149–152]. Furthermore, it has been observed that MTA can increase cytokine release, including IL-1α, IL-1β, IL-2, IL-6, and IL-8, from mineralizing cells and this mild and acute material-induced inflammatory response may also contribute to clinical repair [153–155].

To better characterise the molecular response of the pulp tissue during caries, we have undertaken high-throughput transcriptional profiling using disease and healthy pulp tissue. Data indicated that the predominant tissue processes, pathways, and molecular interactive networks detected were

proinflammatory in nature, while there was minimal evidence of repair-associated molecular events [11] (Figure 3). Indeed, increased expression of many well-characterised proinflammatory mediators was detected while further data-mining enabled us to identify expression changes in several molecules previously not associated with dental tissue disease. We subsequently speculated that underlying molecular repair-related responses may be occurring and, therefore, further bioinformatically interrogated our datasets and identified the candidate repair-related molecule, adrenomedullin (ADM). This pleiotropic cytokine was upregulated during dental disease and is reported to have antibacterial and immunomodulatory properties, as well as being a known molecular mediator of angiogenic and mineralized tissue reparative processes. Others have also shown that it is able to modulate inflammation at the molecular level [156–159]. Our subsequent studies went on to demonstrate that ADM may exert similar effects within the dental tissues and is archived within the dentin during primary dentinogenesis [160]. These data indicate that this molecule may be a viable target for use in future biological therapies for both hard and soft tissue repair of the dentin-pulp complex.

While it is aimed at identifying molecular modulators of dental tissue inflammation, which may have efficacy in enabling hard tissue repair, it is also interesting to speculate that direct delivery of mesenchymal stem cells (MSCs) or their secretomes may provide a novel approach to control inflammation. Indeed, adult/postnatal MSCs, including dental pulp stem cells, isolated from a range of tissues have demonstrable immune-modulatory capability either via their cell-cell contact or via their secreted components which can inhibit proliferation, cytokine/antibody secretion, immune cell maturation, and antigen presentation by T cells, B cells, NK cells, and DCs [161–163]. Direct cell-to-cell contact between stem and immune cells is known to elicit secretion of soluble factors such as TGF-β1 and indoleamine-2,3-dioxygenase-1 which subsequently can dampen the immune response. While MSCs may provide a cell therapy approach to aid repair of inflamed dental tissue if delivered appropriately, better characterization of their secreted active components may enable identification of novel molecules for targeted dental tissue repair.

Data now indicate that, during a progressive carious infection, initially it is the odontoblasts which detect the invading bacteria and, subsequently, cells within the pulp core such as resident immune cells, fibroblasts, stem cells, and endothelial cells become involved in the molecular response. Further autocrine and paracrine signalling amplifies the reaction and leads to an increased immune cell infiltration. The elaboration of a plethora of cytokines and chemokines will have resultant consequences for the tissue and its innate repair mechanisms and this milieu is further added to by the signalling molecules released from the dentin matrix itself by the action of bacterial acids [48]. This local cocktail of bioactive molecules will continue to chronically recruit and activate immune cells, which combat the invading bacteria. The relatively high levels of proinflammatory mediators present in the local environment will likely impair any healing events at the cellular and molecular levels. Currently, the application of dental clinical procedures and restorative materials aims to remove the infection, facilitate the resolution of the inflammatory response, and enable repair processes. Notably, attempts are now being made to apply knowledge of the cytokine networks invoked for diagnostic and prognostic purposes. It is envisaged that these data will enable identification of lesions refractory to endodontic treatment due to unresolved chronic inflammation [164].

While diagnostics are being developed based on the characterisation of the inflammatory response, modulators of inflammation have the potential to be used adjunctively to facilitate the healing response and aid restoration longevity. Recent work has demonstrated that dental resin restorative procedures can be supplemented with antioxidants, such as N-acetyl-cysteine (NAC). This supplementation reportedly provides protection to the pulpal cells from ROS generated following resin placement. Interestingly, NAC may also limit the activation of the key ROS activated NF-κB proinflammatory pathway [165] and this modulation may also minimise the inflammatory response, subsequently creating a more conducive environment for tissue repair. More studies in this area may identify other antioxidants and pathways, which may facilitate dental tissue repair responses.

Other work has demonstrated the importance of the modulation of both ROS and reactive nitrogen species (RNS) to facilitate repair. Kim et al. [166] have recently demonstrated that the anti-inflammatory mechanism of exogenously applied PPARγ in activated human dental pulp cells was likely due to the removal of both NO and ROS, which subsequently suppressed both the NF-κB inflammatory and extracellular signal-regulated kinase (ERK) 1/2 signaling pathways. The anti-inflammatory effects of other naturally derived compounds, such as pachymic acid, derived from the mushroom *Formitopsis niagra*, have also been explored. Interestingly, this compound may not only have anti-inflammatory activity, but also appears to be able to promote odontoblast differentiation via activation of the HO-1 pathway. These data further indicate the important interrelationship between inflammation and repair and its potential application for dental disease treatment [167]. Recently, an exciting area relating to the therapeutic application of regulatory microRNAs (miRNAs) has been reported. These miRNA molecules have been shown to be differentially expressed between healthy and diseased dental pulps [168] and work is ongoing within the pharmaceutical industry to engineer these molecules for delivery to treat a range of inflammatory diseases. Potentially, miRNAs may therefore one day be applied in the treatment of dental disease as a means to tip the balance from a chronic inflammatory environment to one more conducive for tissue repair. It is now evident that more studies are required which target the interactions between the inflammatory and regenerative responses within the dentin-pulp complex as these may identify novel therapies for dental tissue repair.

4. Conclusion

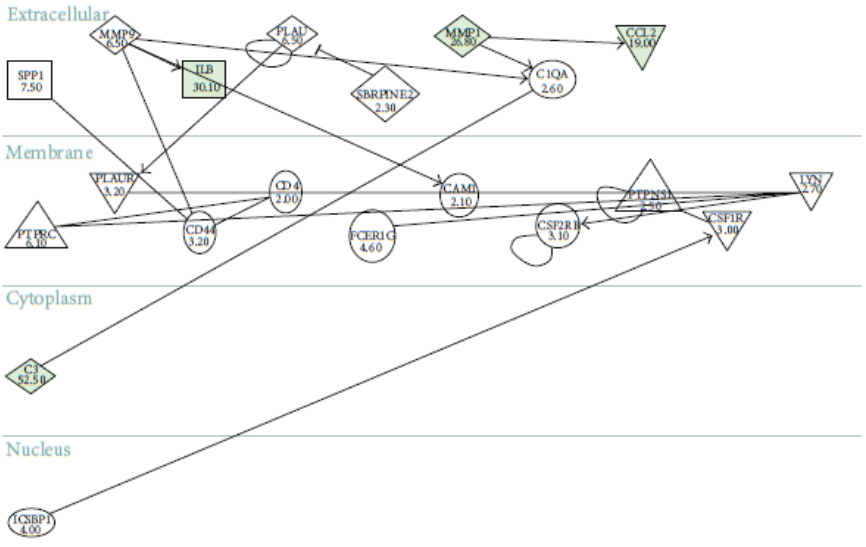
We are now developing a better and more complete understanding of the molecular and cellular events which occur

Function	Network															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Chemotaxis*	■	■	■								■	■		■		
Activation*	■															
Phagocytosis*	■				■											
Development/differentiation*				■												
Respiratory burst/ROS*				■		■		■					■			
Lymphocyte function*					■											■
Apoptosis						■									■	
Calcium mobilization/flux			■		■			■								
Lipid synthesis/metabolism							■	■					■			
Hard tissue formation#									■					■		
Hard tissue resorption#										■			■			
NO synthesis/regulation*													■			
Bone marrow/cell movement*											■			■		

(a) Carious diseased pulp

Function	Network		
	1	2	3
Mitogenesis/cell cycle progression	■	■	■
Cell viability/growth			■
Exocytosis		■	
Cell polarization		■	
Angiogenesis			■

(b) Healthy pulp



(c)

FIGURE 3: Tables ((a) and (b)) showing the key functions associated with the 16 and 3 molecular networks identified as being significantly activated (≥ 6 focus genes) in carious and healthy pulpal tissue, respectively. Shading of boxes indicates the networks which associated with the function and hence supported its inclusion as being active. Analysis was performed using the Ingenuity Pathways Analysis (IPA) software (http://www.ingenuity.com/products/ipa) on the high-throughput datasets reported in McLachlan et al. [11]. Sixteen and three functional categories were identified as being activated in carious diseased and healthy pulpal tissues, respectively. Carious diseased pulp tissue clearly demonstrated increased molecular network and functional activity compared with healthy pulpal tissue. Asterisks (∗) in (a) indicate functions which are associated with immune system cells (as identified by IPA); notably some evidence of hard tissue repair function was also evident (#). Ontological functions identified in (b) likely associate with pulp tissue homeostatic processes. Image (c) shows an example network (network 1 from the carious pulp tissue dataset) which also shows the subcellular localisation of the molecules that were identified as differentially expressed. The activation of this network via intracellular signalling cascades results in the elaboration of key inflammatory-associated chemokines, such as CXCL8 (IL-8) and CCL2, and the matrix metalloproteinases (MMPs) 1 and 9.

in the dentin-pulp complex during inflammation and repair following carious disease. While disinfection of the dental tissue is clearly imperative for the health of the tooth, the subsequent interaction between dental tissue defence and repair is complex and the fine-tuning of the regulation of these processes is important for ensuring which response predominates when vital pulp tissue can be clinically retained or regenerated. It is clear that sustained research activity in this area combined with clinical translational approaches may result in the development of new therapeutics which enable host defence and repair events. Advances in our understanding of the interactions between immune and regenerative responses may therefore influence clinical practice and benefit dental patients in the future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jean-Christophe Farges, Brigitte Alliot-Licht, and Paul R. Cooper contributed equally to this work and should be considered co-first authors.

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1.4 Discussion à partir de l'article 1

Lorsque les bactéries cariogènes pénètrent dans la dentine, des phénomènes de défense se mettent en place pour protéger le tissu pulpaire. Ils comprennent une réaction immunitaire/inflammatoire et une phase de dentinogenèse réactionnelle et/ou réparatrice :

- La réaction immunitaire/inflammatoire (Figure 8) :

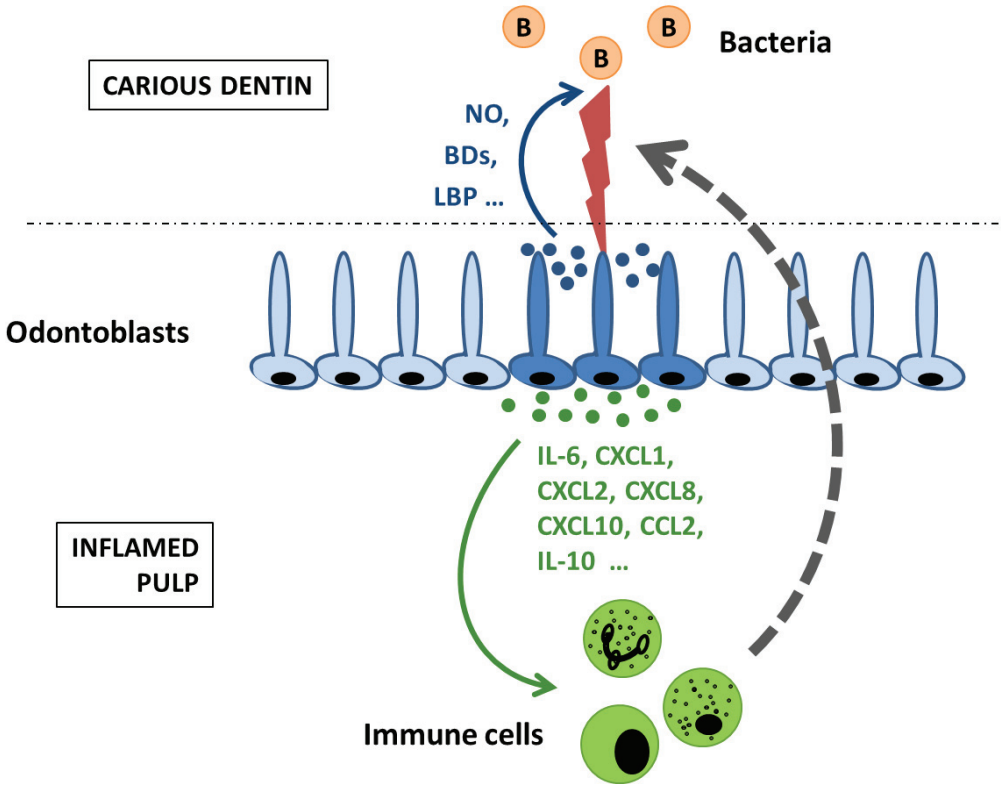

Figure 8 : Aspects clés de la défense des odontoblastes contre les bactéries qui envahissent la dentine. Les bactéries (B) présentes dans la lésion carieuse dentinaire libèrent des composants pathogènes qui activent (flèche rouge) les odontoblastes proches de la lésion (bleus foncés), déclenchant la production de molécules antibactériennes (ronds bleus). Ces molécules diffusent dans les tubules dentinaires pour détruire les microorganismes (oxyde nitrique [NO], bêta-défensines [BDs]) ou diminuer considérablement leur pathogénicité (Protéine de liaison du lipopolysaccharide [LBP]) En parallèle, des médiateurs pro-inflammatoires ou immunomodulateurs (ronds verts), incluant IL-6, IL-10, CXCL1, CXCL2, CXCL8 (IL-8),CXCL10, et CCL2, sont sécrétés par les odontoblastes au pôle opposé et diffusent dans la pulpe sous-odontoblastique (flèche verte) où ils activent et mobilisent diverses populations de cellules immunitaires qui assurent l'immunosurveillance du tissu. Ces dernières migrent alors (flèche grise pointillée) vers l'interface pulpe-dentine sous la lésion pour combattre les bactéries et coordonner la réponse immunitaire.

L'invasion progressive de la dentine par les bactéries cariogènes issues de la cavité buccale déclenche une réaction immunitaire et inflammatoire dans la pulpe sous-jacente, destinée à combattre l'agresseur microbien. De par leur situation à la périphérie pulpaire et la présence d'un long prolongement cellulaire dans la dentine, les odontoblastes sont les premières cellules rencontrées par les microorganismes qui pénètrent dans la dentine. La reconnaissance de ces microorganismes par des récepteurs spécifiques présents à la surface odontoblastique active la synthèse de molécules antibactériennes comme les bêta-défensines et la production de cytokines et de chimiokines pro-inflammatoires qui vont activer les cellules immunitaires dans la pulpe proche de la lésion. Dans les cas d'agression sévère, on assiste à une réduction importante de la synthèse de matrice dentinaire par les odontoblastes, mais lorsque l'agression reste modérée, les odontoblastes gardent une capacité de synthèse dentinaire pour former une barrière destinée à éloigner le tissu pulpaire de l'agresseur. Cette barrière peut être formée de dentine réactionnelle et/ou réparatrice.

Au cours de notre doctorat, nous avons également contribué à démontrer que les odontoblastes activés sont capables de produire de l'oxyde nitrique qui possède une activité antibactérienne (Article 5, en annexe).

- Les dentinogenèses réactionnelle et/ou réparatrice (Figure 9)

Si l'agression de la pulpe dentaire par les bactéries cariogènes reste limitée dans le temps, par exemple grâce à l'éviction de la lésion carieuse par le praticien, elle ne déclenche qu'une réaction immunitaire et inflammatoire modérée. Des phénomènes de cicatrisation, de réparation et de régénération tissulaire vont alors se mettre en place dans la pulpe. Le retour de la pulpe à l'état physiologique est favorisé par la formation d'une barrière de dentine dite tertiaire dans la zone pulpaire proche de la dentine lésée. Cette barrière a pour but d'éloigner le tissu pulpaire de la lésion (bactéries, matériau d'obturation coronaire) afin de favoriser sa cicatrisation. La barrière dentinaire est dite « réactionnelle » lorsqu'elle est d'origine odontoblastique. La dentinogenèse réactionnelle consiste en l'obturation des tubules par de la dentine sclérotique et en l'accroissement de l'épaisseur de la couche de dentine résiduelle située entre les odontoblastes et la dentine lésée par les bactéries (Simon et al. 2008). Si la réponse inflammatoire est plus importante, elle provoque une stase sanguine périphérique et une hypoxie tissulaire qui entraîne la mort des odontoblastes. Dans ce cas, des cellules

mésenchymateuses souches et/ou progénitrices vont se multiplier dans la région sous-odontoblastique et venir au contact de la dentine dans la zone de nécrose. Elles s'y différencient en odontoblastes, dits de remplacement ou de deuxième génération, et déposent alors une dentine tertiaire dite « réparatrice » destinée, comme la dentine réactionnelle, à éloigner le tissu pulpaire de la lésion dentinaire et à favoriser ainsi son retour à l'homéostasie.

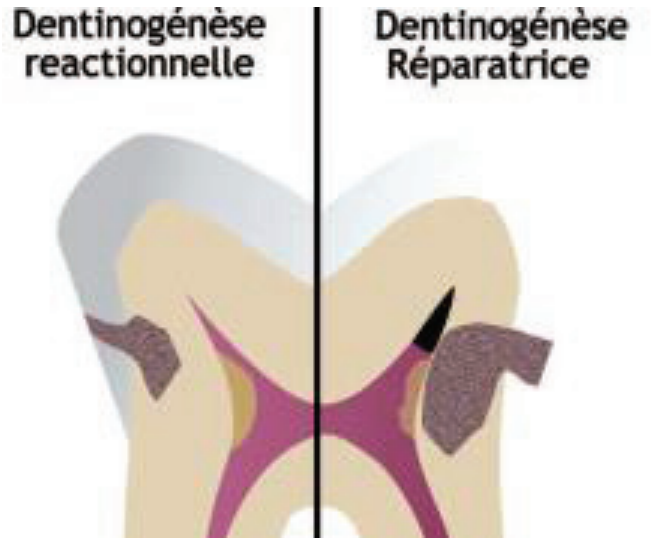

Figure 9 : Les deux types de dentinogenèse tertiaire.
(Simon *et al.* 2008)

Comme dans la moelle osseuse, le tissu adipeux ou le cordon ombilical, la pulpe dentaire contient des CSM et/ou cellules progénitrices en très faible quantité (<1%) (Gronthos et al. 2000)(Waddington et al. 2009)(Sloan & Waddington 2009). Leur localisation et leur mécanisme de recrutement restent hypothétiques (Huang et al. 2009)(Huang 2011)(About 2011)(Chmilewsky et al. 2014) (Figure 10). Il est de plus en plus largement admis que les populations de CSM sont constituées, *in vitro* et *in vivo*, d'un mélange de sous-populations au sein desquelles se trouvent des cellules « souches », des cellules progénitrices, des cellules périvasculaires et des fibroblastes en proportions variables (Huang et al. 2009)(Huang et al. 2010)(Lv et al. 2014)(Harrington et al. 2014).

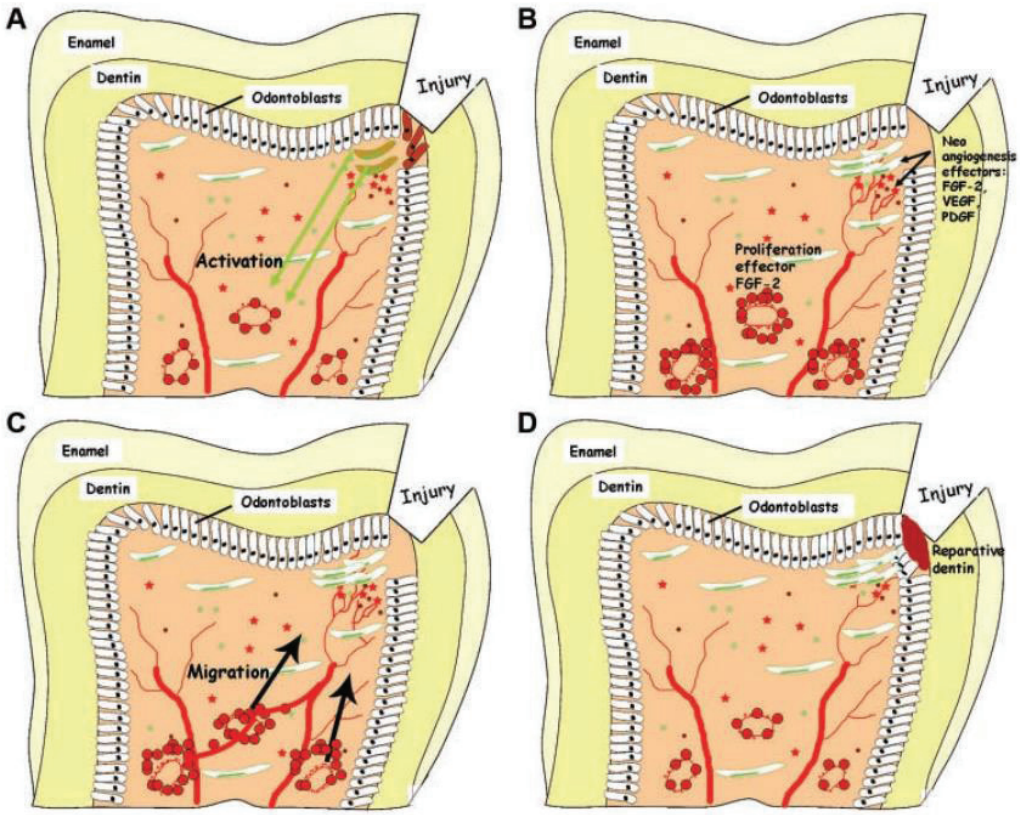

Figure 10 : Hypothèse du recrutement des cellules progénitrices qui forment la dentine tertiaire réparatrice. Lors d'une atteinte carieuse profonde avec exposition pulpaire, les odontoblastes primaires sont détruits (A), entrainant la libération de facteurs de croissance et le recrutement de cellules souches/progénitrices pulpaires (B et C). Au contact de la lésion, les cellules progénitrices se différencient en odontoblastes de deuxième génération et déposent de la dentine réparatrice (D) (About 2011)**.**

Certains auteurs restent toutefois prudents sur la capacité des cellules souches/progénitrices pulpaires à former une vraie dentine, car des observations cliniques récentes réalisées chez l'homme indiquent que le tissu synthétisé serait plus proche du tissu osseux que du tissu dentinaire (Ricucci et al. 2014)**.**

D'une manière générale, il existe deux types de réactions pulpaires de la pulpe face à une lésion carieuse profonde :

- Lorsque l'inflammation pulpaire reste modérée, si un traitement est effectué par le praticien, et qu'un biomatériau étanche est placé en regard de la pulpe après éviction du tissu carié et désinfection de la cavité amélo-dentinaire, le tissu va le plus souvent réduire son processus inflammatoire et une phase de cicatrisation et de réparation va être initiée (Tran et al.

2012)(Riccuci et al., 2014). Cela permet à la pulpe dentaire de **retourner à un état « physiologique » (Situation A, figure 11).**

- Dans le cas contraire, le plus souvent en l'absence d'élimination du tissu amélo-dentinaire infecté par le praticien, les barrières de dentine tertiaire mises en place lors des mécanismes de défense pulpaires sont elles-mêmes détruites et rapidement dépassées. Ceci entraîne une inflammation plus importante qui ne permet pas le retour de la pulpe à l'état « physiologique ». En effet, dans ce cas, l'inflammation pulpaire s'étend à l'ensemble du tissu, devient irréversible du fait de la stase sanguine importante et de l'hypoxie qui l'accompagnent, et il est alors nécessaire de réaliser l'**exérèse partielle (pulpotomie) ou complète (pulpectomie) de la pulpe pour éviter sa nécrose (Situation B, figure 11).**

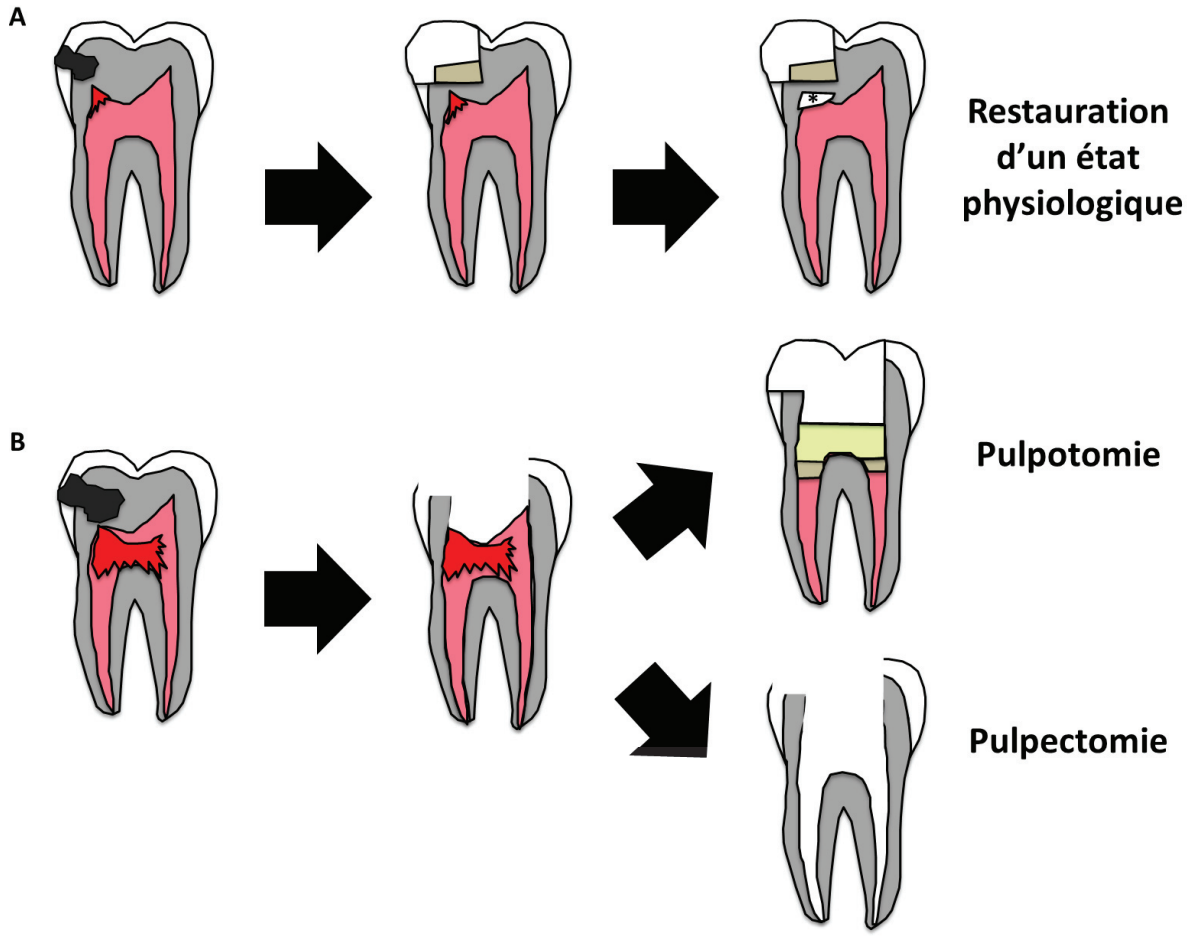

Figure 11 : Réaction pulpaire face à une lésion carieuse profonde. (A) Dans le cas où l'inflammation pulpaire est modérée, l'éviction carieuse diminue fortement l'inflammation et une barrière de dentine tertiaire se met en place (*). (B) Si l'inflammation pulpaire devient irréversible, une ablation du tissu, partielle (pulpotomie) ou complète (pulpectomie), doit être réalisée.

Si la pulpotomie représente un traitement de choix pour les dents permanentes immatures, sa généralisation aux dents matures n'en est encore qu'au stade expérimental (Villat et al. 2013)(Simon et al. 2013).

Lorsqu'une pulpectomie est réalisée, il faut ensuite maintenir l'étanchéité de la dent vis à vis de la cavité buccale pour éviter une colonisation bactérienne. Ceci est réalisé en obturant l'espace endodontique à l'aide de gutta-percha (stratégie conventionnelle ou traditionnelle). Récemment, une autre stratégie d'obturation, plus biologique, a été proposée (Figure 12).

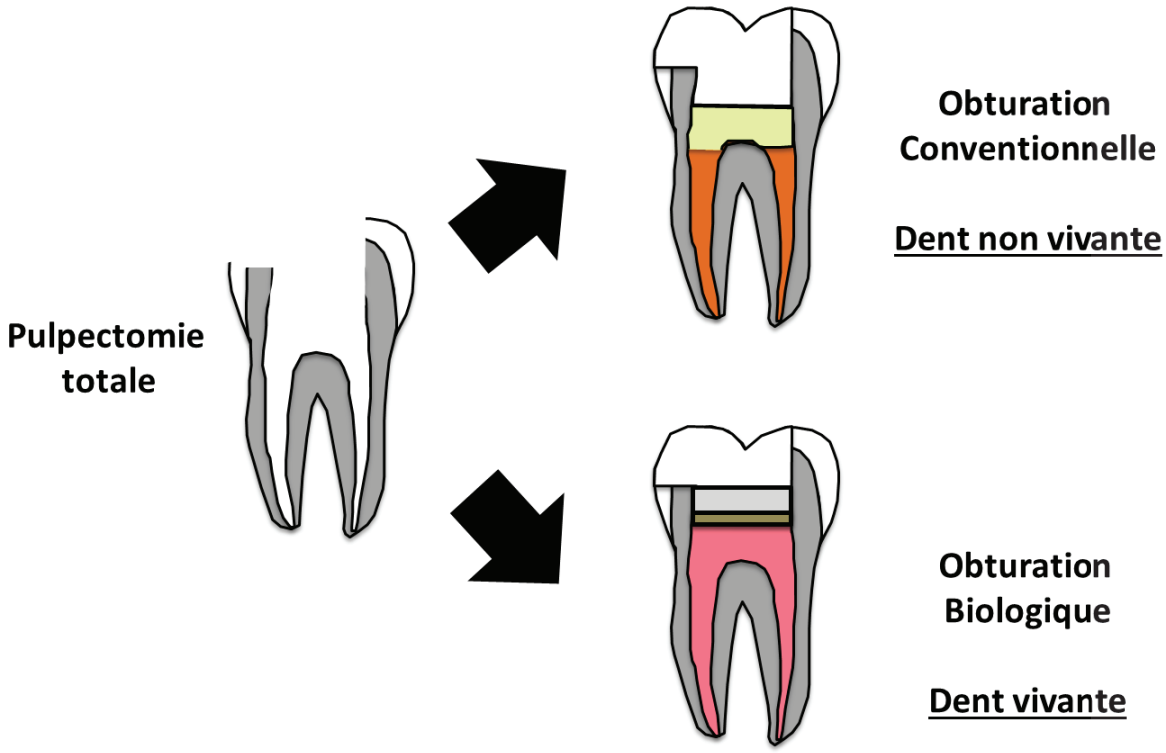

Figure 12 : Les deux stratégies d'obturation endodontique après réalisation d'une pulpectomie.

2. Les stratégies d'obturation endodontique

2.1 La stratégie actuelle : l'obturation endodontique conventionnelle à la gutta-percha

En cas d'atteinte irréversible de l'ensemble de la pulpe suite à une carie ou un traumatisme, il est nécessaire de réaliser l'exérèse complète du tissu pulpaire. Suite à cette éviction, l'espace endodontique est désinfecté pour éliminer les bactéries et les toxines qu'il contient, puis est mis en forme mécaniquement et obturé de la manière la plus étanche possible avec de la gutta-percha scellée aux parois dentinaires avec du ciment (Huang et al. 2008)(Huang 2009). La gutta-percha est un matériau relativement biocompatible mais non résorbable. Il est thermoplastique, ce qui permet de l'ajuster à la forme de l'endodonte. Sa composition est la suivante : environ 20% de gutta-percha pure, 35 à 75% d'oxyde de zinc, de 2 à 30% sulfate de baryum, et des agents plastifiants, des colorants et des agents antimicrobiens.

L'obturation de l'endodonte à la gutta-percha présente un certain nombre de limites. En effet, l'analyse des traitements endodontiques montre des taux d'échecs variables, parfois importants pour les praticiens non-spécialistes, notamment en France. En effet, ces traitements montrent de nombreux défauts d'étanchéité, de contamination bactérienne de l'endodonte, ou de présence d'une pathologie infectieuse ou inflammatoire d'origine endodontique dans la région périapicale (Boucher et al. 2002)(Tavares et al. 2009). Une pathologie périapicale peut être à l'origine de douleurs chroniques qui sont accrues lors de la mastication. L'anatomie radiculaire souvent complexe rend souvent difficile la préparation mécanique et chimique de l'endodonte (Robinson et al. 2012). De plus, la mise en forme mécanique des canaux radiculaires est un acte invasif qui expose aux risques de fracture instrumentale et de coloration postopératoire de la dent. Elle s'accompagne également de risques de complications infectieuses et/ou inflammatoires en cas de dépassement instrumental ou du matériau d'obturation au niveau du sinus maxillaire ou du nerf mandibulaire (Figure 13)(Simon et al. 2013).

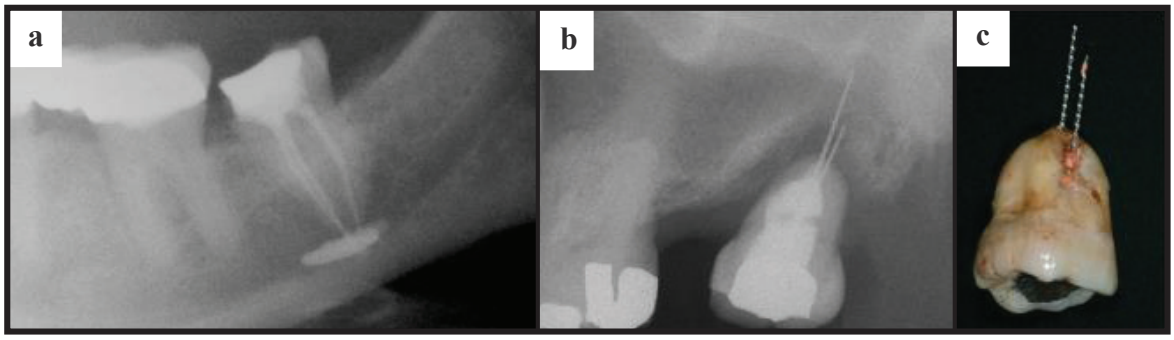

Figure 13 : Risques per et post opératoires lors de la réalisation d'un traitement endodontique. (a) L'obturation des dents mandibulaires peut entrainer la diffusion du matériau au niveau du nerf mandibulaire. (b) La compaction de la gutta-percha expose à des fractures instrumentales qui peuvent nécessiter l'extraction de la dent (c) (Gluskin 2009)***.***

Sur le plan mécanique, la résistance d'une dent dont l'endodonte a été obturé par la gutta-percha diminue, ce qui augmente le risque d'apparition de fissures ou de fractures au niveau de l'émail et de la dentine résiduels (Kishen & Vedantam 2007)(Dietschi et al. 2008)(Soares et al. 2008)(Zelic et al. 2014). Egalement, comme l'un des motifs principaux de consultation chez un chirurgien-dentiste est l'apparition d'une douleur dentaire, la perte de la proprioception et du système de défense immunitaire dans la pulpe masque très souvent l'évolution des pathologies des dents dévitalisées et diminue considérablement leur temps de survie sur l'arcade. Enfin, une dent dévitalisée présente plus de risques de développer une lésion périapicale qui est une porte d'entrée potentielle dans l'organisme pour un essaimage bactérien par le réseau vasculaire (Friedman et al. 2003). C'est pour cette raison que l'obturation de l'endodonte à la gutta-percha est contre-indiquée chez les personnes à haut risque d'endocardite infectieuse (ANSM 2011).

Pour pallier ces inconvénients, la recherche s'intéresse à la mise au point de méthodes alternatives, plus fiables et plus sûres, ayant pour objectif d'obturer l'endodonte de manière plus « biologique » en remplaçant le tissu pulpaire par un tissu structuralement et fonctionnellement semblable.

Deux stratégies ont été proposées pour obturer l'endodonte de manière biologique : la revascularisation et l'ingénierie tissulaire.

2.2 La revascularisation

2.2.1 Historique

L'histoire de la revascularisation débute dans les années 60, lorsque Nygaard-Østby rapporte que la formation d'un caillot sanguin dans l'endodonte suite à l'irritation du périapex permet d'obtenir une « cicatrisation » pulpaire. L'auteur observe la disparition des signes pathologiques au niveau périapical et, dans certains cas, des signes radiographiques de fermeture du foramen apical (Nygaard-Ostby & Hjortdal 1971). D'un point de vue histologique, les structures formées dans l'endodonte ressemblent à un mélange de zones fibreuses contenant des cellules fibroblastiques et de zones de minéralisation semblables à celles trouvées dans le cément cellulaire.

Cette approche de revascularisation a été délaissée pendant près de 30 ans, puis sont apparues des publications rapportant les premiers cas de revascularisation de dent permanente immature suite à l'application d'une procédure standardisée. Le protocole consiste toujours à induire la formation d'un caillot sanguin au sein de la lumière canalaire pour permettre une colonisation cellulaire de l'endodonte, mais il nécessite au préalable de désinfecter complètement l'endodonte avec une pâte antibiotique pendant plusieurs semaines. A l'issue de la désinfection, un saignement apical est induit par irritation mécanique du périapex afin de remplir l'endodonte avec un caillot sanguin. Un matériau dentino-inducteur est ensuite placé au contact de ce caillot pour permettre la formation d'une barrière de dentine tertiaire (Banchs & Trope 2004)(Trope 2008).

A ce jour, des centaines de revascularisations de dents permanentes immatures ont été réalisées avec succès chez l'homme, permettant la revitalisation de l'endodonte et la poursuite de l'édification radiculaire des dents immatures (Diogenes et al. 2013)(Moreno-Hidalgo et al. 2014).

2.2.2 Protocole

Pour obtenir une revascularisation de l'endodonte, un protocole clinique en deux étapes est le plus souvent proposé (Figures 14 et 15)(Simon 2010)(Moreno-Hidalgo et al. 2014).

Schématiquement, lors de la première séance sont réalisés :

- une anesthésie avec vasoconstricteur et la pose du champ opératoire,

- l'accès au canal et la mise en place d'une pâte antibiotique (généralement à base de métronidazole, de ciprofloxacine et de minocycline) ou d'hydroxyde de calcium,
- l'obturation provisoire de la cavité d'accès.

Deux à trois semaines plus tard, lors de la seconde séance sont réalisés :

- une anesthésie sans vasoconstricteur,
- l'élimination de la pâte antibiotique intracanalaire et le rinçage du canal,
- le franchissement du foramen apical par des limes endodontiques pour induire un saignement dans l'endodonte à partir du périapex et la formation d'un caillot intracanalaire,
- l'obturation de la partie coronaire du canal avec un biomatériau dentino-inducteur,
- l'obturation définitive de la cavité d'accès par un matériau type résine composite.

Le patient est ensuite suivi tous les 3 mois.

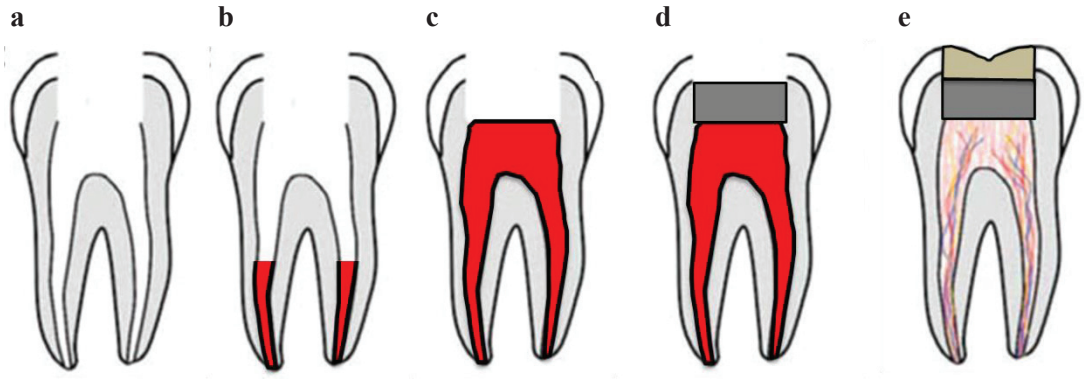

Figure 14 : Représentation schématique du protocole de revascularisation. Après exérèse du tissu pulpaire (a), un saignement est induit dans les canaux (b) et la chambre pulpaire (c), puis un matériau (d) est placé dans l'endodonte pour permettre aux cellules de se différencier. Un matériau de reconstitution coronaire est ensuite mis en place pour isoler la pulpe régénérée du milieu buccal (e).

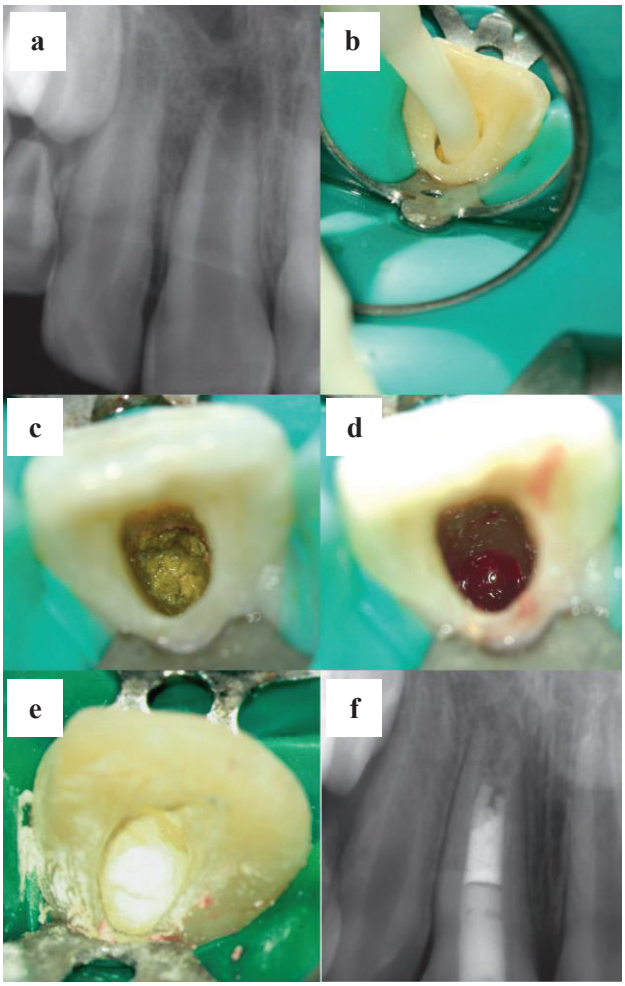

Figure 15 : Etapes de la revascularisation endodontique. (a) Radiographie préopératoire d'une incisive centrale immature (11) dont la pulpe est nécrosée et qui présente une lésion apicale. (b) Mise en place d'une pâte contenant les trois antibiotiques. (c) Vue clinique de la médication dans le canal. (d) Vue clinique du canal rempli de sang. (e) Vue clinique du bouchon de MTA® (Mineral trioxide aggregate®) placé au contact du caillot sanguin. (f) Radiographie de contrôle à 10 mois postopératoire. (Simon 2010)

2.2.3 Analyse histologique

Plusieurs semaines après une procédure de revascularisation, l'analyse histologique montre que le canal radiculaire contient un tissu très vascularisé similaire à une papille apicale (Huang et al. 2008). Plusieurs études ont permis d'observer trois tissus au sein de l'endodonte revascularisé: un tissu cémentoïde, un tissu osseux et un équivalent de ligament parodontal (Figure 16)(Huang 2009)(Wang et al. 2010)(Chen et al. 2012). D'autres ont rapporté des résultats différents avec, soit l'absence de tissu dur dans le canal, soit la présence d'une couche odontoblastique au contact de la dentine (Shimizu et al. 2012). Cette présence pourrait s'expliquer par la persistance de tissu pulpaire vivant dans le canal, tissu qui pourrait participer

à la régénération de la pulpe (Huang et al. 2013). Aujourd'hui, plusieurs hypothèses tentent d'expliquer le phénomène de revascularisation endodontique et de cicatrisation/allongement radiculaire :

- 1) Des cellules présentes dans la papille apicale ont survécu à la nécrose pulpaire grâce à l'apport sanguin au niveau de cette papille (Huang et al. 2008). Ces cellules prolifèrent et se différencient localement, provoquant l'allongement de la racine et l'épaississement de la paroi dentinaire (maturation radiculaire).

- 2) Une partie des CPD de l'espace endodontique radiculaire est restée vivante, et suite à la revascularisation, contribue à la maturation de la racine.

- 3) Au contact du microenvironnement endodontique et dentinaire, des cellules du caillot sanguin se multiplient et se différencient en fibroblastes, en odontoblastes ou en cémentoblastes (Lovelace et al. 2011).

- 4) Des cellules du ligament parodontal prolifèrent dans le périapex, migrent à l'intérieur du canal radiculaire, puis se différencient et déposent un tissu minéralisé sur la paroi dentinaire interne, au niveau du tiers inférieur de la racine jusqu'à l'apex. C'est l'hypothèse la plus probable aujourd'hui.

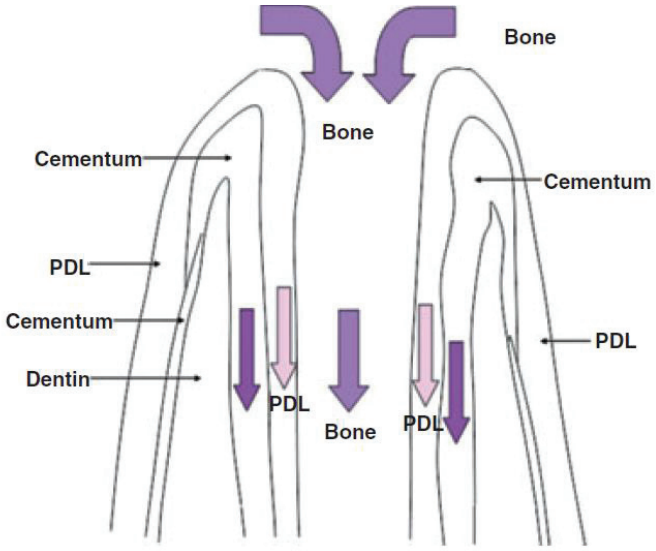

Figure 16 : Formation intracanalaire de tissu osseux (Bone), de tissu cémentoïde (Cementum) et de ligament parodontal (PDL) au niveau du tiers radiculaire apical lors d'une procédure de revascularisation. (Huang 2009)

Ces observations laissent penser que la structure régénérée au sein du canal est plus proche d'un tissu parodontal que du tissu pulpaire d'origine. Ceci peut s'expliquer par le recrutement de cellules de la région périapicale qui vont se différencier dans l'endodonte revascularisé en cellules parodontales et non en fibroblastes pulpaires ou en odontoblastes.

Dans ce contexte, il est impossible d'affirmer aujourd'hui que le tissu formé au sein du canal après un traitement de revascularisation soit de la pulpe dentaire (Nosrat et al. 2011)(Neha et al. 2011)(Andreasen & Bakland 2012)(Cao et al. 2015)**.**

2.2.4 Données cliniques

L'analyse des données cliniques s'avère difficile du fait des multiples protocoles de recrutement des patients, de débridement de l'endodonte, de suivi des patients, ou du fait de la composition de la pâte intracanalaire utilisée comme médication temporaire. Afin de réduire cette diversité, certains organismes comme l'association américaine d'endodontie (AAE) commencent à proposer des protocoles standardisés de revascularisation. La mise en place d'essais cliniques randomisés est également indispensable pour démontrer définitivement le bénéfice apporté par cette thérapeutique.

2.2.5 Limites et perspectives

Avant de proposer cette thérapeutique à l'ensemble des praticiens, ou pour l'adapter aux dents permanentes matures, il existe de nombreux objectifs à atteindre, parmi lesquels :

- L'obtention d'une désinfection endocanalaire qui n'entraîne pas de toxicité cellulaire. En effet, il semble exister un risque de toxicité après l'utilisation de la triade antibiotique, ainsi que des risques de coloration de la dentine (Figure 17)(Ding et al. 2009)(Kim et al. 2010)(Simon 2010). Le développement d'une stratégie de désinfection moins agressive apparait ainsi indispensable. L'utilisation d'hydroxyde de calcium, moins toxique, notamment pour les cellules du périapex, et qui ne semble pas entrainer de coloration dentinaire, est une voie prometteuse (Albuquerque et al. 2014)(Cao et al. 2015).

Figure 17 : Coloration de la couronne d'une dent revascularisée après utilisation de la pâte tri-antibiotique. (Ding et al. 2009)(Kim et al. 2010)

- L'utilisation d'une solution d'irrigation canalaire qui permette la différenciation odontoblastique. En effet, la matrice dentinaire contient de nombreuses molécules bioactives qui facilitent la différenciation odontoblastique des cellules qui viennent à son contact. Ainsi, le remplacement de l'hypochlorite de sodium, qui détruit les protéines, par l'acide éthylène-diamine-tétracétique (EDTA), chélatant du calcium qui démasque les molécules de la matrice dentinaire sans les endommager, favorise l'adhésion, la migration et la différenciation des cellules du tissu régénéré en odontoblastes (Huang et al. 2010)(Galler et al. 2011)(Galler et al. 2015)(Cao et al. 2015).

- La détermination des limites des indications thérapeutiques. Aujourd'hui, la technique de revascularisation est indiquée uniquement pour les dents immatures des patients jeunes (entre 8 et 13 ans), car plusieurs études suggèrent qu'elles possèdent un potentiel de régénération et de cicatrisation pulpaire important (Thomson & Kahler 2010)(Wigler et al. 2013). Une étude récente indique toutefois que cette technique pourrait aussi être utilisée chez des patients plus âgés (Wang et al. 2015).

- La détermination du diamètre apical minimal permettant la formation d'un caillot intracanalaire. De nombreuses études expérimentales et rétrospectives ont démontré que la revascularisation d'une dent humaine est possible si le diamètre du foramen apical est supérieur à 1,1 mm (Huang 2009)(Huang et al. 2013). Cependant, d'autres études ont déterminé que la limite inférieure du diamètre du foramen pour permettre la revascularisation est de 0,70 mm (Iohara et al. 2013)(Law 2013).

- La définition précise des objectifs thérapeutiques. L'Association Américaine des Endodontistes (AAE) a défini 3 objectifs lors des procédures de revascularisation :

1. l'élimination des symptômes cliniques et l'obtention de signes de guérison osseuse,
2. l'augmentation de l'épaisseur et/ou de la longueur radiculaire,
3. une réponse positive aux tests de vitalité.

Toutefois, au vu des données histologiques, les réponses obtenues aux tests de vitalité doivent être interprétées avec prudence, car l'épaisseur du matériau de restauration peut empêcher la transmission du stimulus thermique jusqu'au tissu vivant régénéré présent à l'intérieur du canal.

- La comparaison du devenir à long terme des dents traitées par revascularisation avec celui des dents obturées de manière conventionnelle. Actuellement, il n'existe pas de travaux qui comparent de manière satisfaisante et à long terme la survie des dents traitées avec cette thérapeutique par rapport à celle des dents traitées avec la technique conventionnelle à base de gutta-percha (Moreno-Hidalgo et al. 2014). Or, l'édification radiculaire par cémentogenèse ou ostéogenèse, et non par dentinogenèse, pourrait modifier les propriétés mécaniques de la dent. De plus, l'ostéogenèse ou la cémentogenèse intrapulpaires pourraient avec le temps conduire à l'obturation complète du canal radiculaire et compliquer ainsi la réalisation des futurs traitements endodontiques (Chen et al. 2012)(Lin et al. 2014).

En conclusion, si cette technique est intéressante pour revasculariser l'endodonte des dents permanentes matures, elle présente pour l'instant un niveau de preuve et de recul trop limité pour permettre sa généralisation (Moreno-Hidalgo et al. 2014).

Dans ce contexte, l'ingénierie tissulaire peut être une alternative.

2.3 La régénération de la pulpe dentaire par ingénierie tissulaire

2.3.1 Principe et définition de l'ingénierie tissulaire

L'ingénierie tissulaire est un domaine interdisciplinaire qui cherche à restaurer et à maintenir la fonction d'un tissu en appliquant les principes de la biologie et de l'ingénierie. Le principe est de développer des équivalents tissulaires (*Tissue equivalents*) en associant des cellules, un biomatériau et des facteurs biochimiques, de telle sorte que les cellules puissent proliférer et se différencier dans un environnement proche de leur environnement naturel. Il est nécessaire, au préalable, de collecter, d'isoler et d'amplifier les cellules à partir d'une biopsie du patient ou d'un donneur compatible. L'objectif est de recréer un tissu hors du corps afin d'implanter un dispositif « sur-mesure » chez le patient.

L'objectif de l'ingénierie tissulaire étant de recréer un tissu similaire sinon identique à l'original, la connaissance des mécanismes de régulation qui président au développement embryonnaire des tissus et organes est capitale. Dans ce contexte, l'ingénierie du complexe pulpodentinaire humain aura pour objectif de donner aux cellules ensemencées dans le biomatériau un environnement 3D qui permette la différenciation des cellules centrales en fibroblastes, des cellules périphériques en odontoblastes, et la colonisation de l'ensemble du tissu par les vaisseaux sanguins, les fibres nerveuses et les cellules immunocompétentes (Figure 18).

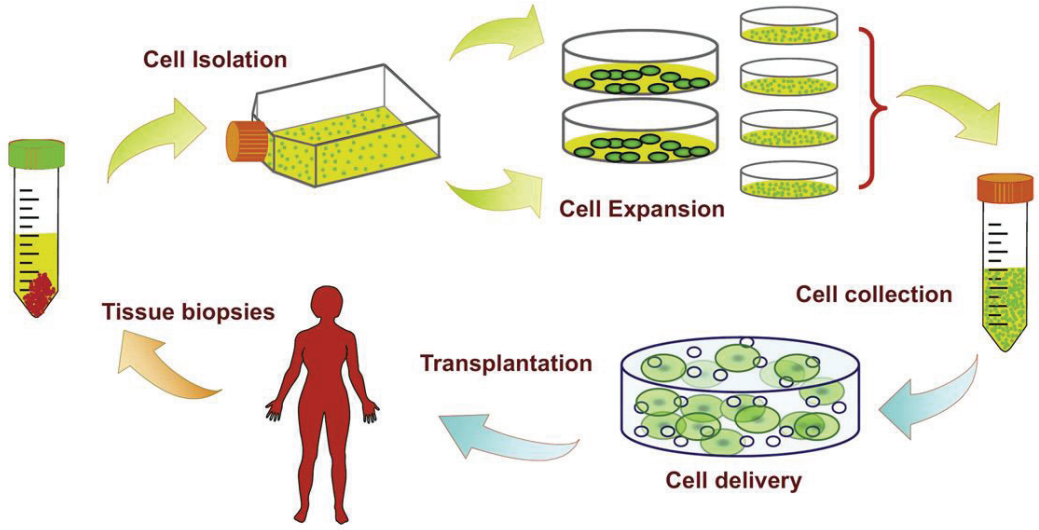

Figure 18 : Représentation schématique de l'ingénierie tissulaire. (Chen et al. 2011)

2.3.2 Etats des lieux de la recherche sur l'ingénierie de la pulpe dentaire

L'ingénierie tissulaire de la pulpe dentaire consiste à régénérer le tissu pulpaire en implantant dans l'endodonte un biomatériau contenant ou non des cellules. La pulpe dentaire est considérée comme un tissu difficile à régénérer car elle a une organisation cellulaire et tissulaire complexe **(cf. 1.1)**, ainsi qu'une situation anatomique qui complique la gestion de l'inflammation apicale et de l'angiogenèse qui se développe dans l'endodonte à partir de l'apex. Dans ce contexte, plusieurs stratégies d'ingénierie de la pulpe dentaire ont été mises en place. Chacune essaie de reconstruire un tissu pulpaire *ad integrum*, une « pulpe équivalente », en associant différents cocktails de molécules bioactives, de biomatériaux et de cellules (Figure 19)(Murray et al. 2007)(Rosa et al. 2013)(Dissanayaka et al. 2014)(Cao et al. 2015).

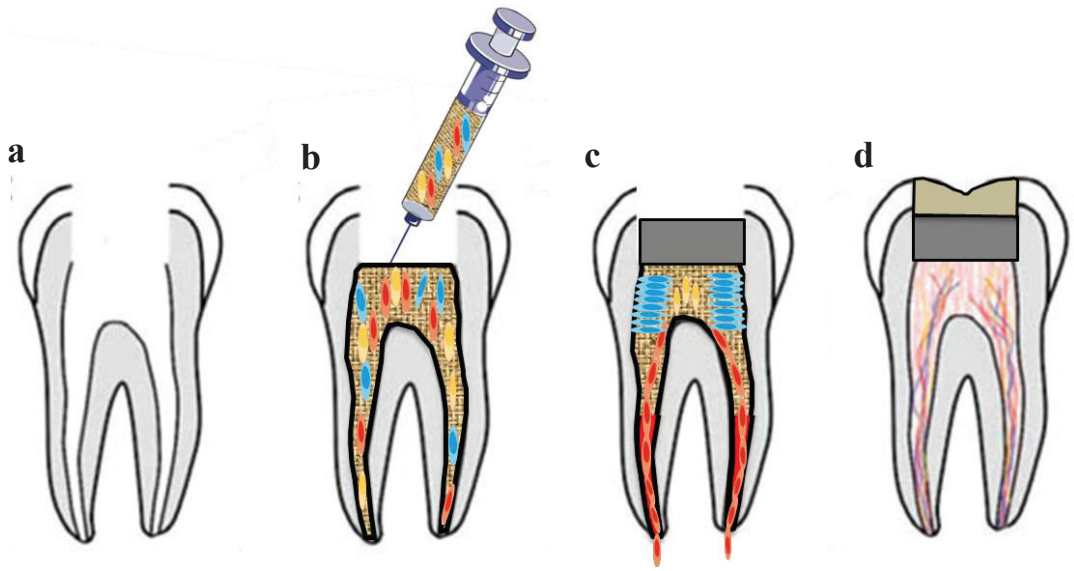

Figure 19 : Représentation schématique du concept d'ingénierie de la pulpe dentaire. Après éviction du tissu pulpaire lésé, désinfection et mise en forme de l'endodonte (a), un mélange de cellules, biomatériau et facteurs de croissance est implanté (b), puis un matériau dentino-inducteur est placé au contact du mélange injecté pour promouvoir la différenciation odontoblastique (c). Parallèlement à la dégradation et/ou au remodelage du biomatériau, les cellules s'organisent pour recréer un tissu pulpaire fonctionnel vascularisé, innervé, immunocompétent, et capable de former de la dentine au contact du matériau dentino-inducteur (d).

Une revue de la littérature résumant ces différentes stratégies a été réalisée récemment. Chaque article a été classé selon trois critères : le type de biomatériau utilisé, les différentes CSM et les molécules bioactives (Figure 20)(Albuquerque et al. 2014).

Author/Year	Study Design	Scaffold	Bioactive Molecules	Stem Cells	Most Relevant Findings
Dobie *et al.*, 2002	*In vitro*	Alginate HY with TGF-β1	Yes	No	✓ Release of TGF-β1; ✓ odontoblast-like cell differentiation
Galler *et al.*, 2008	*In vitro*	PA self-assembling NF - HY	No	Yes (DPSCs and SHEDs)	✓ Easy to handle; ✓ introduced into small defects; ✓ cell proliferation
Cordeiro *et al.*, 2008	*In vivo*	PLLA	No	Yes (SHEDs)	✓ Pulp-like tissue formation
Prescott *et al.*, 2008	*In vivo*	Col Type I with CP and DMP-1	No	Yes (DPSCs)	✓ New pulp-like tissue formation and organization
Ishimatsu *et al.*, 2009	*In vitro*	Gelatin HY incorporation of FGF-2	Yes	No	✓ Release of FGF-2 ✓ Induces the invasion of dental pulp cells and vessels
Yang *et al.*, 2010	*In vitro* *In vivo*	NF-PCL/gelatin/nHA	No	Yes (DPSCs)	✓ DPSC differentiation toward an odontoblast-like cells *in vitro* and *in vivo*
Feng *et al.*, 2010	*In vitro*	NF-PLGA/PLLA scaffolds with DOX	Yes	No	✓ Release of DOX; ✓ inhibition of bacterial growth for a prolonged duration
Huang *et al.*, 2010	*In vivo*	poly-D,L-lactide/glycolide	No	Yes (DPSCs and SCAPs)	✓ Pulp-like tissue formation with vascularity and dentin-like structure
Nakashima and Iohara, 2011	*In vivo*	Col with SDF-1	No	Yes (dog pulp $CD105^+$, $CD31^-$ SP cells)	✓ Complete pulp-like tissue regeneration
Galler *et al.*, 2011b	*In vivo*	GF–laden peptide HY with VEGF, TGFβ -1, and FGF-2	Yes	No	✓ Release of VEGF, TGF-β1, and FGF2; ✓ odontoblast-like cell differentiation; ✓ pulp-like tissue formation
Galler *et al.*, 2011a	*In vivo*	PEGylated fibrin gel	No	Yes (DPSCs, SHEDs, PDLSCs, and BMSSCs)	✓ All types of dental stem cells proliferated; ✓ excellent biocompatibility; ✓ insertion into small defects
Wang *et al.*, 2011	*In vitro* *In vivo*	NF-PLLA	No	Yes (DPSCs)	✓ Attachment, proliferation, and differentiation of human DPSCs;
Iohara *et al.*, 2011	*In vivo*	Col with SDF-1	No	Yes (dog pulp $CD105^+$ cells)	✓ Complete pulp-like and dentin-like tissue regeneration; ✓ orthotopic model
Galler *et al.*, 2012	*In vitro*	Self-assembling MDP Peptide NF-HY	Yes	Yes (DPSCs)	✓ Pulp-like tissue formation
Zhang *et al.*, 2012	*In vivo*	DDM-PLLG/Co–CS–HA	No	Yes (DPSCs)	✓ Potential as attractive scaffolds for odontogenic differentiation
Ishizaka *et al.*, 2012	*In vivo*	Col with SDF-1	No	Yes (dog pulp, BM, Adipose $CD31^-$ SP cells)	✓ Complete pulp-like tissue regeneration; ✓ orthotopic model
Akkouch *et al.*, 2013	*In vitro*	3D Col/HA/PLCL	No	Yes (DPSCs)	✓ DPSC differentiation and proliferation
Bottino *et al.*, 2013	*In vitro*	NF PDS II-with MET and CIP	Yes	No	✓ Release MET or CIP; ✓ antimicrobial activity against *Ef* and *Pg*
Bottino *et al.*, 2014b	*In vitro*	NF PDS II-HNTs	No	No	✓ Potential in the development of a bioactive scaffold for regenerative endodontics
Cavalcanti *et al.*, 2013	*In vitro*	Self-assembling peptide HY (Puramatrix™)	No	Yes (DPSCs)	✓ DPSC survival, proliferation, and differentiation
Coyac *et al.*, 2013	*In vitro*	3D dense Col HY	No	Yes (SHEDs)	✓ Odontogenic cell differentiation and mineralization
Iohara *et al.*, 2013	*In vivo*	Col with G-CSF	No	Yes (dog mobilized DPSCs)	✓ Complete pulp-like and dentin-like tissue regeneration; ✓ orthotopic pre-clinical model
Murakami *et al.*, 2013	*In vivo*	Col	No	Yes (human mobilized DPSCs)	✓ Ectopic model; ✓ pulp-like tissue regeneration
Rosa *et al.*, 2013	*In vivo*	Peptide HY (Puramatrix™) with rhCol type I	No	Yes (SHEDs)	✓ SHED injected into full-length human root canals differentiate into functional odontoblasts
Yang *et al.*, 2014	*In vivo*	Porous chitosan/col scaffold	Yes	Yes (DPSCs)	✓ Release of BMP-7 gene; ✓ DPSC differentiation into odontoblast-like cells *in vitro* and *in vivo*
Iohara *et al.*, 2014	*In vivo*	Col with G-CSF	No	Yes (dog mobilized DPSCs)	✓ Orthotopic model; ✓ less volume of regenerated pulp-like tissue in aged dogs compared with that in young dogs
Nakashima and Iohara, 2014	*In vivo*	No	No	Yes (mobilized DPSCs)	✓ Complete pulp-like tissue regeneration with thick coronal dentin formation in pulpectomized root canals

Figure 20 : Résumé des différentes stratégies d'ingénierie tissulaire en endodontie (voir aussi liste des abréviations). (Albuquerque et al. 2014)

Il semble qu'à l'heure actuelle, plusieurs combinaisons permettent de reconstruire une « pulpe équivalente » *in vitro* et *in vivo* par ingénierie tissulaire. Ainsi, les travaux de l'équipe de Huang ont montré la formation de tissu minéralisé suite à l'implantation de cellules parodontales dans la chambre pulpaire de la canine de chien (Huang 2009). Une autre équipe a obtenu la régénération d'une pulpe dentaire vascularisée et innervée à partir de différentes sources de CSM de la pulpe dentaire (Iohara et al. 2013).

Si certains biomatériaux sont encore à un stade expérimental de leur développement, d'autres semblent déjà répondre aux exigences d'une future application clinique pour régénérer la pulpe dentaire. Par exemple, les solutions injectables comme les hydrogels semblent être appropriées cliniquement grâce à leur facilité de mise en place dans l'endodonte et à leur biocompatibilité. Concernant les molécules bioactives, les BMPs, les FGFs ou le SDF-1 apportent une aide intéressante car elles favorisent la différenciation des CPD en odontoblastes. Leur cinétique de relargage est un élément-clé pour régénérer une pulpe dentaire et non pas un tissu osseux (Albuquerque et al. 2014)**.**

2.3.3 Limites et perspectives

Au regard des données actuelles, l'ingénierie tissulaire de la pulpe dentaire est encore à un stade expérimental. En effet, si la preuve de concept a bien été établie dans des modèles *in vitro* et *in vivo* chez l'animal, il n'existe pas encore de résultats publiés chez l'homme. Un essai clinique vient juste d'être initié au Japon (https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000008691&language=E), mais les résultats ne sont pas encore connus (Nakashima & Iohara 2014).

Pour développer des protocoles d'ingénierie tissulaire de la pulpe dentaire, des études supplémentaires sont nécessaires (Schmalz & Smith 2014), notamment pour :

- Déterminer la population cellulaire idéale. Outre la pulpe dentaire, il existe de nombreux tissus contenant des CSM, comme par exemple la moelle osseuse, le tissu adipeux et le cordon ombilical. Toutefois, l'un des défis dans la régénération des tissus humains par ingénierie tissulaire est l'obtention de cellules à partir d'une source peu ou non invasive pour

le patient, ce qui tend à exclure les CSM de la moelle osseuse prélevées par ponction. De plus, la capacité des CSM d'origine non-dentaire à régénérer un complexe pulpodentinaire reste à démontrer. A l'inverse, de nombreuses études ont démontré la capacité des CPD, souches ou progénitrices, à se différencier en odontoblastes *in vitro* et *in vivo*. Cependant, les populations de cellules souches ou progénitrices de la pulpe dentaire sont en fait très hétérogènes, constituées d'un mélange de sous-populations au sein desquelles se trouvent des cellules souches mésenchymateuses (CSM), des cellules progénitrices, des péricytes et des fibroblastes en proportions variables (Huang et al. 2009)(Huang et al. 2010)(Lv et al. 2014)(Harrington et al. 2014).

Dans ce contexte, la caractérisation précise de ces cellules par immunophénotypage et tri cellulaire est nécessaire afin de définir et sélectionner la ou les populations les plus appropriées pour la reconstruction d'un complexe pulpodentinaire.

- Contrôler la dissémination des cellules pulpaires implantées. Une des inquiétudes de l'ingénierie tissulaire est de savoir ce que deviennent les cellules implantées chez les patients. Une étude récente a toutefois montré, dans un modèle de régénération pulpaire chez le rat, l'absence de diffusion dans l'organisme des cellules implantées dans la pulpe dentaire (Souron et al. 2014).

- **Développer un biomatériau facilement utilisable et mimant la matrice extracellulaire de la pulpe.** Il existe de nombreux biomatériaux qui permettent la survie des CPD, mais tous ne sont pas pertinents pour régénérer un complexe pulpodentinaire (Hilkens et al. 2015). En effet, le bon biomatériau devra permettre l'attachement, la prolifération et la différenciation en odontoblastes des CPD situées à la périphérie du biomatériau. Certaines technologies aujourd'hui prometteuses, comme par exemple la bio-impression tissulaire, pourraient se révéler difficiles à mettre en place cliniquement ou à industrialiser.

- Optimiser la cinétique de vascularisation et d'innervation de l'équivalent pulpaire obtenu. L'obtention d'un réseau vasculaire fonctionnel est la clé de l'ingénierie tissulaire, car il permet au tissu d'être viable (Novosel et al. 2011). Des études récentes ont rapporté le potentiel pro-angiogénique des CPD humaines placées dans un environnement hypoxique, confirmant ainsi leur intérêt pour l'ingénierie pulpaire (Kerkis & Caplan 2012)(Janebodin et al. 2013)(Hilkens et al. 2013)(Bronckaers et al. 2013).

- Gérer l'inflammation et la cicatrisation post-opératoire provoquée par la greffe tissulaire et la dégradation du biomatériau. La greffe d'un nouveau tissu entraine chez l'hôte une réaction inflammatoire d'intensité variable, dépendant de la nature et de l'origine des CSM greffées, ainsi que des produits de dégradation du biomatériau dans lequel les CSM sont ensemencées (Hilkens et al., 2015).

Dans ce contexte, les CSM ont des propriétés immunosuppressives qui dépendent de plusieurs facteurs parmi lesquels la source à partir de laquelle les cellules sont isolées, le nombre de passages réalisés pour les amplifier, la quantité de cellules implantées et les conditions pathologiques spécifiques de l'hôte (Schmalz & Smith 2014). Ces propriétés ne sont pas constitutives mais sont induites par les cytokines pro-inflammatoires présentes dans le microenvironnement comme l'interféron-gamma ou le tumor necrosis fator-alpha en association avec l'interleukine-1beta. Ces cytokines déclenchent en effet la production par les CSM de nombreuses molécules immunorégulatrices comme le TGF-beta, l'indoleamine 2,3-dioxygenase (IDO), l'oxyde nitrique (NO), le TNF-inducible gene-6 (TSG6), la prostaglandine-E2 et l'interleukine-10. Chez l'homme, plusieurs études ont montré le rôle prédominant de l'IDO, un enzyme qui catalyse la dégradation du tryptophane. L'immunosuppression serait ainsi due à la déplétion en tryptophane qui résulte de l'activation de cet enzyme (Wang et al. 2014). Le potentiel immunosuppresseur des CSM a été confirmé pour les cellules souches/progénitrices de pulpe dentaire, ce qui constitue pour ces cellules un atout majeur dans l'optique de futurs projets de régénération de la pulpe dentaire (Pierdomenico et al. 2005)(Wada et al. 2009)(Ma et al. 2014).

Il faut toutefois noter que le potentiel immunomodulateur des CSM dépend du type et de l'intensité des stimuli inflammatoires : les CSM sont en effet immunosuppressives dans les cas de forte inflammation mais immunostimulatrices dans les cas de faible inflammation (Wang et al. 2014).

- Développer des protocoles industriels permettant de produire des cellules comme sont produits les médicaments. Jusqu'à présent, les CPD sont obtenues dans des conditions de culture respectant peu les bonnes pratiques de fabrication définies par les textes règlementaires internationaux (voir plus loin). Elles sont notamment isolées dans des conditions de stress pouvant modifier leurs propriétés biologiques et cultivées dans des milieux contenant

du sérum et en présence de produits xénogéniques potentiellement contaminés par des microorganismes, et donc dangereux pour l'homme.

Même si ces conditions de préparation cellulaire sont actuellement tolérées par les autorités de régulation internationales, il est nécessaire de développer des protocoles alternatifs pour une production plus efficace, reproductible, sure et standardisée, à la manière dont sont produits les médicaments (Tirino & Papaccio 2012)(La Noce et al. 2014)(Nakashima & Iohara 2014)(Mayo et al. 2014)(Albuquerque et al. 2014)**.**

3. Développement d'un médicament de thérapie innovante

Le développement et la production d'une nouvelle thérapeutique pour la régénération de la pulpe dentaire (biothérapie) nécessite de fabriquer un « médicament » contenant des cellules. En Europe, ce type de médicaments est dit « de thérapie innovante » (MTI) et doit être produits au sein d'établissements pharmaceutiques en respectant des règles de Bonnes Pratiques de Fabrication (BPF). L'établissement doit avoir obtenu une Autorisation de Mise sur le Marché (AMM) délivrée par la commission européenne après évaluation par l'Agence Européenne des Médicaments (EMA).

3.1 Définition des biothérapies

Les biothérapies regroupent l'ensemble des thérapies cellulaires, tissulaires ou géniques utilisant soit des organismes vivants (gènes, cellules, tissus,…), soit des substances prélevées à partir d'organismes vivants. Selon l'article 8 de loi n°2011-322 du 22 mars 2011 déclinant le règlement européen n° 1394/2007, les biothérapies sont définies pour trois grands types de produits, chacun relevant d'un cadre réglementaire spécifique :

- **Les préparations de thérapie cellulaire (PTC)**. Ce sont des produits cellulaires ou tissulaires (allogéniques ou autologues) à finalité thérapeutique et soumis à des modifications **non substantielles**. Ces préparations ne sont pas des médicaments au sens du code de la santé, mais sont des produits de santé sous la compétence de l'Agence Nationale de Sécurité du Médicament (ANSM). Elles sont réglementées au niveau national sur la base de la directive 2004/23/CE.

- **Les médicaments de thérapie innovante (MTI).** Cette définition couvre les médicaments de thérapie génique, de thérapie cellulaire somatique, issus de l'ingénierie tissulaire et cellulaire, et les médicaments combinés de thérapie innovante (associant un MTI avec un dispositif médical). Les cellules subissent des modifications **substantielles** et/ou ont une fonction différente de leurs fonctions d'origine. Les MTI suivent le

règlement européen n°1394/2007 et sont d'une part régulés au niveau national pour les essais cliniques et au niveau européen pour leur mise sur le marché et l'ensemble des procédures de suivi post-autorisation.

- **Les médicaments de thérapie innovante préparés ponctuellement (MTI-PP).** Ce sont des MTI fabriqués et utilisés au sein d'un seul état membre. Ils sont préparés de façon ponctuelle au sein d'un même établissement pour un patient donné. Les MTI-PP sont sous le régime des MTI du règlement européen, mais sont exemptés de la clause de l'AMM centralisée et doivent suivre un cadre réglementaire national équivalent aux règles communautaires applicables en matière de qualité et de sécurité.

L'un des éléments importants de cette classification repose sur la notion de modifications « substantielles » apportées au cours de la production des cellules (Figure 21). Si le produit subit l'une de ces modifications, il est considéré comme un MTI (ou un MTI-PP) et ne pourra pas être une préparation (EMA/CAT/600280/2010). La notion de modification substantielle doit se comprendre comme une modification des propriétés biologiques ou comme une modification conduisant à une utilisation différente de sa fonction initiale. Une succession de modifications, chacune non substantielle, peut aussi conduire à un procédé qui modifie les propriétés des cellules/tissus et constituer au final une modification substantielle (Figure 22)(Ancans 2012)(Chabannon et al. 2014).

Préparations de thérapie cellulaire Manipulations non substantielles*
Découpage Broyage Façonnage Centrifugation Trempage dans des solutions antibiotiques ou antimicrobiennes Stérilisation Irradiation Séparation, concentration ou purification de cellules Filtration Lyophilisation Congélation Cryoconservation Vitrification
Médicaments de thérapie innovante Manipulations substantielles**
Culture *ex vivo* Expansion/activation *ex vivo* Manipulation génétique (transfert de gènes, modification du génome) Altération du phénotype

Figure 21 : Procédés d'ingénierie cellulaire permettant de distinguer les PTC et les MTI. * Liste non exhaustive mentionnée à l'annexe 1 du règlement CE n° 1394/2007. **Manipulations généralement considérées comme substantielles par le « Committee for advanced therapies » dans ses recommandations scientifiques (Chabannon et al. 2014)**.**

	Critères de classification du produit thérapeutique	Échelle de production	Exportation	Réglementation applicable	Bonnes pratiques applicables	Infrastructure assurant la production et la distribution
Préparations de thérapie cellulaire (PTC)	Manipulation non substantielle et réinjection/ réimplantation homologue	Restreinte	Possible	Nationale Transcription des directives tissus/cellules	Bonnes pratiques tissus/cellules*	Unité de thérapie cellulaire/ banque de tissus
Médicaments de thérapie innovante préparés ponctuellement (MTI-PP)	Manipulation substantielle ou réinjection/ réimplantation non homologue	Restreinte	Interdite	Européenne, nationale	Bonnes pratiques de fabrication (médicaments) (adaptées ?)	Établissements pharmaceutiques ou autres disposant d'une autorisation spécifique
Médicaments de thérapie innovante	Manipulation substantielle ou réinjection/ réimplantation non homologue	Variable : de médicaments orphelins jusqu'à des médicaments destinés à traiter des pathologies fréquentes	Possible	Européenne	Bonnes pratiques de fabrication (médicaments)	Établissements pharmaceutiques

Figure 22 : Principales caractéristiques applicables aux trois catégories de produits thérapeutiques à usage humain issus de l'ingénierie cellulaire ou tissulaire. * Décision du 27 octobre 2010 définissant les règles de bonnes pratiques relatives à la préparation, à la conservation, au transport, à la distribution et à la cession des tissus, des cellules et des préparations de thérapie cellulaire (Chabannon et al. 2014)**.**

Ainsi, un produit cellulaire comme les CSM répond plus naturellement à la définition d'un MTI si son application est à visée hétérologue et avec une commercialisation à grande échelle. Sinon, il sera un MTI-PP destiné à une utilisation autologue, et son caractère « ponctuel », adapté à un « patient déterminé », et non destiné à sa fonction essentielle chez le receveur.

Dans le cadre de l'ingénierie tissulaire de la pulpe dentaire, les cellules et/ou CSM appartiennent à la famille des biothérapies et des MTI/MTI-PP. Cependant, toute cellule ou tissu préparé, transformé ou conservé avec un objectif thérapeutique, entre dans le cadre de la règlementation française et européenne au sein de sous-catégories des MTI.

3.2 Les médicaments de thérapie innovante (MTI)

Dès lors que des cellules sont partiellement ou entièrement récupérées d'un corps humain avec pour objectif d'être utilisées de manière thérapeutique, elles deviennent un MTI. Les MTI sont définis comme des préparations cellulaires (*Advanced-Therapy Medicinal Products* [ATMPs]) par les règlementations française et européenne et sont répartis en 4 catégories (Figure 23)(Pacini 2014)(Source ANSM) :

3.2.1 Les médicaments de thérapie génique

Le « médicament de thérapie génique » ou *Gene Therapy Medicinal Product* (GTMP) doit répondre à deux conditions :

a) Il doit contenir ou être constitué d'un acide nucléique recombinant administré à des personnes en vue de réguler, de réparer, de remplacer, d'ajouter ou de supprimer une séquence génétique.

b) Son effet thérapeutique, prophylactique ou diagnostique, dépend directement de la séquence d'acide nucléique recombinant qu'il contient ou du produit de l'expression génétique de cette séquence.

3.2.2 Les préparations de thérapie cellulaire somatique

La « préparation de thérapie cellulaire somatique » ou *somatic Cell Therapy Medicinal Product* (sCTMP) présente les caractéristiques suivantes :

a) Elle contient ou consiste en :

- des cellules ou tissus qui ont fait l'objet **d'une manipulation substantielle** pour en modifier les caractéristiques biologiques, les fonctions physiologiques ou les propriétés structurales,
- ou des cellules ou tissus qui ne sont pas destinés à être utilisés pour la ou les mêmes fonctions essentielles chez le receveur et le donneur.

Et

b) Elle est présentée comme possédant des propriétés permettant de traiter, prévenir ou diagnostiquer une maladie à travers l'action métabolique, immunologique ou pharmacologique de ces cellules ou tissus, ou utilisée chez une personne ou administrée à une personne dans une telle perspective.

Les conditions (a) et (b) sont inclusives.

3.2.3 Les préparations de l'ingénierie cellulaire ou tissulaire

Sont considérés comme "tissus de l'ingénierie cellulaire ou tissulaire " ou *Tissue Engineered Products* (TEP) les cellules ou tissus qui répondent à au moins l'une des conditions suivantes:

a) Les cellules ou tissus ont été soumis à une **manipulation substantielle**, de façon à obtenir des caractéristiques biologiques, des fonctions physiologiques ou des propriétés structurelles **utiles à la régénération, à la réparation ou au remplacement recherchés**.

b) Les cellules ou tissus ne sont pas destinés à être utilisés pour la (les) même(s) fonction(s) essentielle(s) chez le receveur et chez le donneur.

3.2.4 Les préparations combinées de thérapie innovante

Les médicaments de thérapie innovante (médicaments de thérapie cellulaire, thérapie génique ou d'ingénierie tissulaire) ou *Combined Advanced Therapy Products* (CATP) intègrent dans leur composition :

- Un ou plusieurs dispositifs médicaux au sens de l'article 1er, paragraphe 2, point a), de la directive 93/42/CEE,
- Ou bien un ou plusieurs dispositifs médicaux implantables actifs au sens de l'article 1er, paragraphe 2, point c), de la directive 90/385/CEE.

Et

Leur partie cellulaire ou tissulaire doit contenir des cellules ou des tissus viables,

Ou

Leur partie cellulaire ou tissulaire contenant des cellules ou des tissus non viables doit être susceptible d'avoir sur le corps humain une action considérée comme essentielle par rapport à celle des dispositifs précités.

En conclusion, si des cellules (par exemple des CSM) sont produites pour être (Figure 23) :
- transfusées, elles seront **une préparation de thérapie cellulaire somatique.**
- utilisées pour la régénération, la réparation ou le remplacement d'une structure, elles seront **une préparation de l'ingénierie cellulaire ou tissulaire.**
- combinées avec un biomatériau, elles deviendront **une préparation combinée de thérapie innovante.**

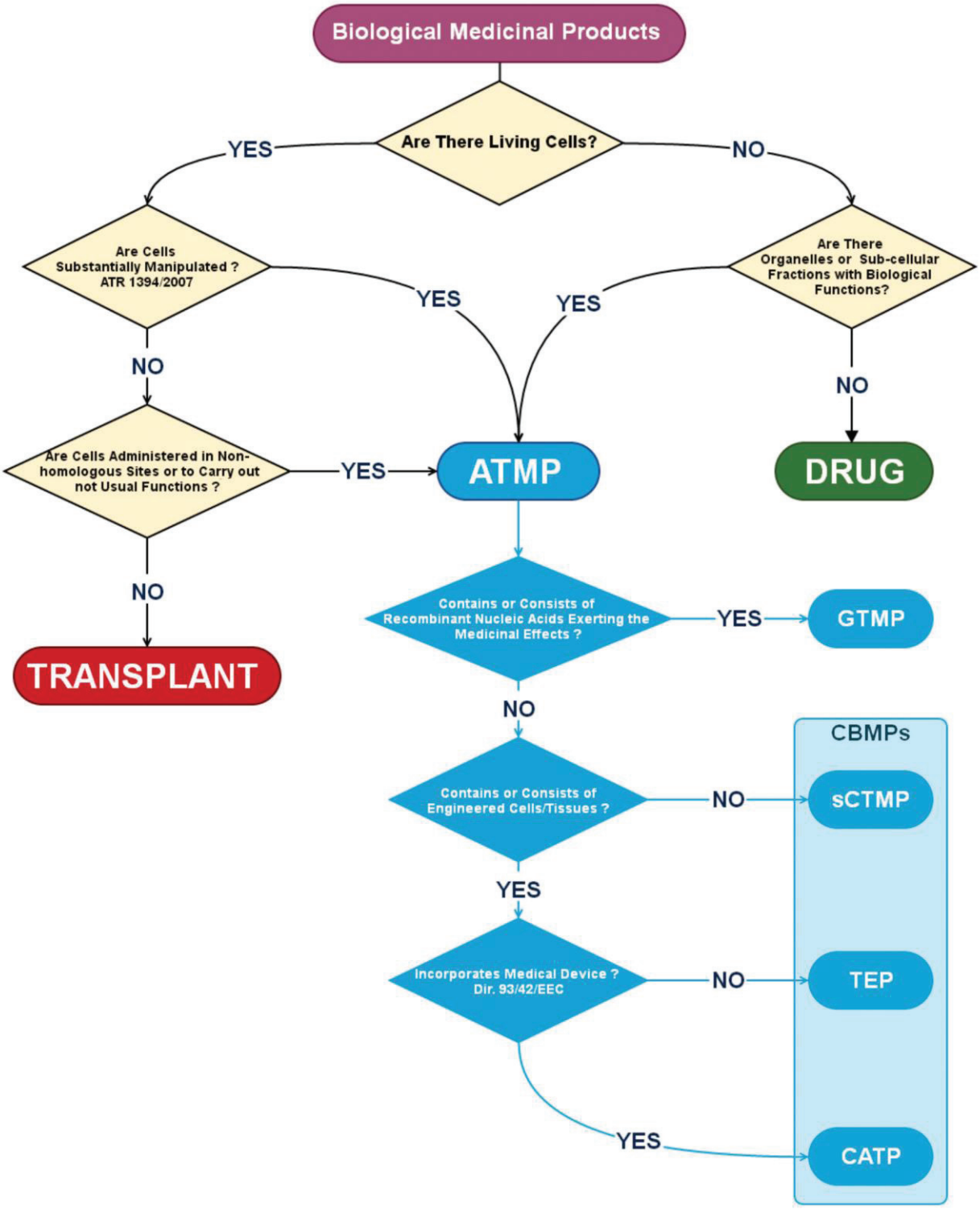

Figure 23 : Algorithme permettant la définition d'un produit médicinal contenant des cellules (Cell-Based Medicinal Product (CBMP). Ces médicaments sont définis comme des préparations cellulaires ou Advanced-Therapy Medicinal Products (ATMPs) et répartis en 4 catégories : un « médicament de thérapie génique » ou Gene Therapy Medicinal Product (GTMP), une « préparation de thérapie cellulaire somatique » ou somatic Cell Therapy Medicinal Product (sCTMP), un « tissu de l'ingénierie cellulaire ou tissulaire » ou Tissue Engineered Product (TEP), un médicament combiné de thérapie innovante ou Combined Advanced Therapy Products (CATP).
(Pacini 2014)

3.3 Les bonnes pratiques de fabrication

Les directives de Bonnes Pratiques de Fabrication (BPF) ont pour objectif de garantir la qualité et la sécurité d'un produit dans toute une gamme d'industries, y compris les industries du médicament. Ainsi, les BPF établissent des normes pour que les produits fabriqués soient d'une efficacité, d'une pureté et d'une qualité conforme à une utilisation clinique.
Différents organismes de régulation dans le monde fournissent des directives BPF, comme l'Agence Européenne des Médicaments (EMA) ou la *Food and Drug Administration* (FDA) américaine. D'autres pays suivent aussi les directives BPF fournies par l'Organisation Mondiale de la Santé (OMS). Ces agences ont pour rôle de veiller à ce que seuls des produits sûrs soient mis sur le marché, mais aussi d'estimer les risques et les avantages d'une nouvelle thérapie.

Ainsi, le parlement européen a édité la régulation EC 1394/2007 pour introduire notamment la notion de produits thérapeutiques à partir de CSM. Ce texte suit en partie les précédentes directives 2003/94/EC et 2002/98/EC sur l'utilisation de produits médicaux chez l'homme et la définition de normes de qualité et de sécurité pour la collecte, le contrôle, la transformation, la conservation et la distribution du sang humain et des composants sanguins. Depuis ce texte, l'EMA édite des directives et des recommandations scientifiques qui visent à fournir une base de réflexion supplémentaire sur les exigences en terme de qualité, de sécurité et d'efficacité pour obtenir une autorisation de mise sur le marché (AMM).
(*http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000298.jsp&murl=menus/regulations/regulations.jsp&mid=WC0b01ac05800862bd*).

La production d'un MTI ou MTI-PP doit ainsi être réalisée en respectant certaines exigences en ce qui concerne :

- le personnel,
- les locaux,
- le matériel,
- les activités,
- le contrôle de la qualité,
- le transport, le conditionnement et l'étiquetage,
- la documentation et les systèmes informatisés,
- la gestion des non-conformités, réclamations et biovigilance.

Ces exigences garantissent que les tissus et les cellules sont préparés, contrôlés, conservés selon des normes de qualité. Elles s'appliquent tout au long de la chaîne, du prélèvement à la distribution. Il est à noter que les directives BPF sont des directives et non des lois, mais de nombreux pays ont adopté des lois qui obligent à suivre les directives BPF à l'échelle locale.

3.4 Le cas des cellules souches/stromales mésenchymateuses (CSM)

Pour démontrer l'efficacité d'une nouvelle thérapeutique, il faut satisfaire de nombreuses exigences comme réaliser des essais cliniques et suivre des démarches administratives et éthiques.

Suite à leur découverte et à la caractérisation de leurs propriétés, les CSM ont été rapidement mises sur le devant de la scène, ce qui a suscité de nombreux espoirs pour les patients. Cependant, une recherche bibliographique rapide sur les essais cliniques et les MTI commercialisés utilisant des CSM fait ressortir que ces thérapeutiques ont encore besoin de faire leurs preuves pour transformer ces espoirs en réalité. En effet, les résultats récents indiquent des bénéfices plutôt modérés et non reproductibles (Allison 2009)(Daley 2012)(Pacini 2014). Ces résultats seraient expliqués en partie par la variabilité des conditions de culture utilisées pour l'isolement et l'amplification *ex vivo*. Cette variabilité pourrait en entrainer une autre, celle des produits cellulaires utilisés qui expliquerait elle-même en partie la variabilité des résultats observés (Allison 2009)(Pacini 2014)(Ma et al. 2014). Par exemple, la différence de composition du sérum présent dans les milieux de culture pourrait impacter sensiblement le phénotype des cellules (Bieback et al. 2009).

Dans le cas des CSM, il existe aussi des recherches qui sont effectuées hors du cadre classique, sans réaliser d'essais cliniques. Ces expérimentations sont réalisées de manière compassionnelle sur de petits groupes de patients ou au sein de cliniques spécialisées dans le tourisme médical. Il convient de rester prudent sur cette approche, car les résultats de ces expérimentations seront difficilement exploitables, étant très susceptibles aux biais de recrutement et d'analyse (Lau et al. 2008)(Daley 2012).
L'une des solutions qui pourrait permettre d'apporter une reproductibilité dans les résultats serait de rationaliser les procédés et d'effectuer les bons contrôles qualité (Romagnoli et al.

2011)(Patrikoski et al. 2013)(Carvalho et al. 2013)(Nguyen et al. 2014). Ainsi, certains auteurs ont proposé des protocoles standardisés pour optimiser la qualité des cellules implantées (Figure 24). Dominici *et al.* en 2006 ont initié cette démarche en proposant des critères pour définir les CSM (Dominici et al. 2006). Cependant, ces critères sont aujourd'hui remis en cause car ils ne sont pas spécifiques et ne permettent pas notamment de discerner de manière standardisé les CSM des fibroblastes ou des péricytes (Russell et al. 2010)(Alt et al. 2011)(Halfon et al. 2011)(Al-Nbaheen et al. 2013). Ainsi, des protocoles plus récents permettent d'envisager une production plus standardisée de CSM, par exemple pour des applications d'immunomodulation (Wang et al. 2012)(Sensebé et al. 2013)(Wuchter et al. 2015). Récemment, une réactualisation des critères pour caractériser les cellules après isolement et amplification a été proposée, afin de mieux définir les CSM qui peuvent être utilisées lors d'une application clinique (Torre et al. 2015).

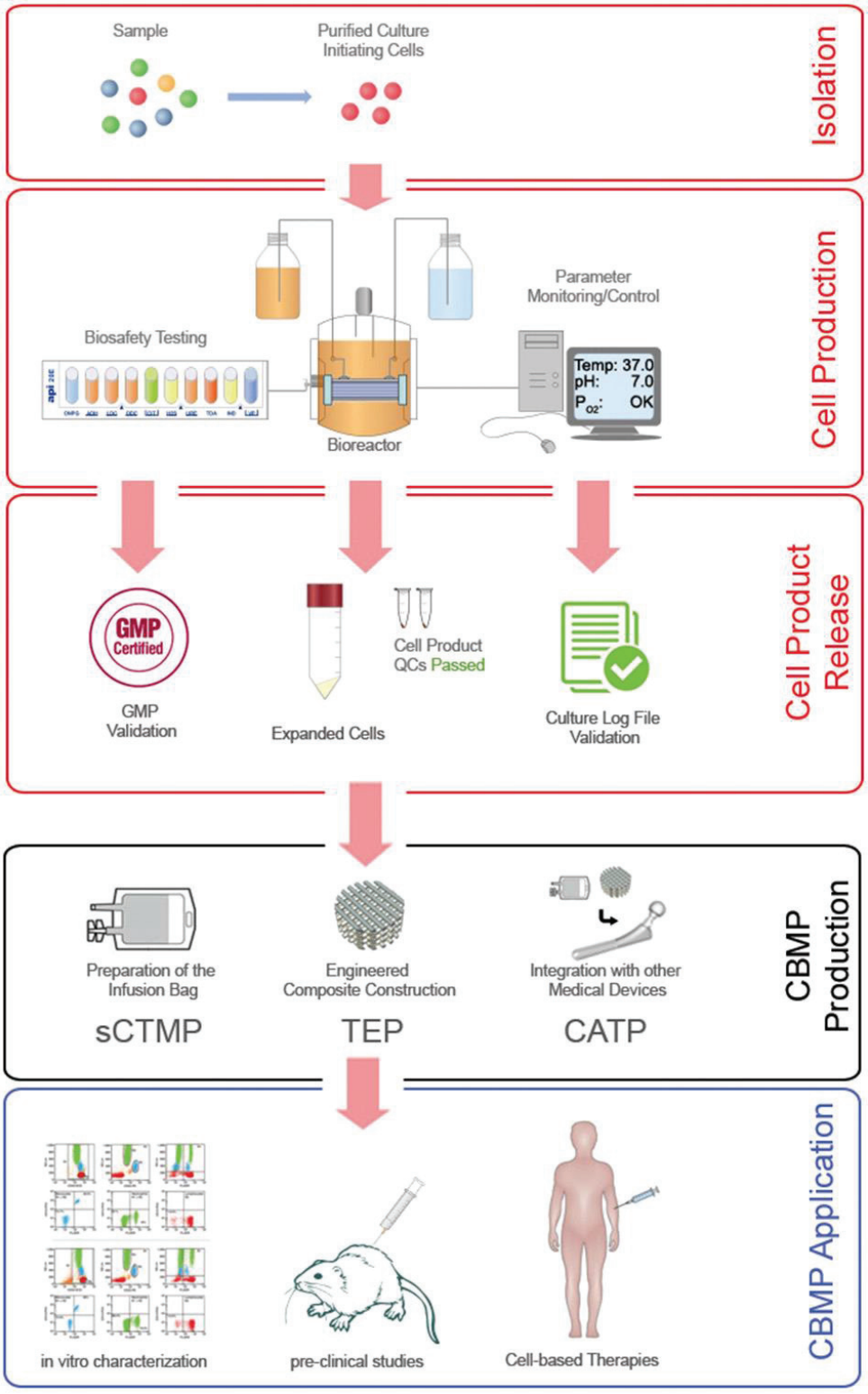

Figure 24 : Représentation schématique des étapes de fabrication d'un médicament de thérapie innovante contenant des cellules, selon une approche respectant les bonnes pratiques de fabrication. Cette approche nécessite de standardiser l'isolement, la production et les étapes de démarche qualité des cellules pour produire un médicament reproductible et utilisable pour la clinique et pour la recherche. (Pacini 2014)

PROBLEMATIQUE ET OBJECTIFS

Comme nous l'avons vu, l'un des défis majeurs de la régénération des tissus humains par ingénierie tissulaire est l'obtention de cellules de qualité, à partir d'une source peu ou non invasive pour le patient. Dans ce contexte, la pulpe des troisièmes molaires présente un grand intérêt car ces dents sont couramment extraites par les chirurgiens-dentistes à la fin des traitements orthodontiques. Le tissu pulpaire contient en effet des cellules mésenchymateuses, les CPD, parmi lesquelles on trouve une proportion non négligeable de cellules souches/progénitrices (CSM), qui peuvent être isolées, amplifiées en culture, et dont le potentiel de différenciation a été plusieurs fois démontré (Gronthos et al. 2000)(Huang et al. 2009).

Toutefois, ces cellules sont obtenues actuellement dans des conditions respectant peu les BPF et un vrai effort d'amélioration et de standardisation est à réaliser lors de la production de CPD afin de s'assurer de la qualité du « produit cellulaire » final (Tirino & Papaccio 2012)(La Noce et al. 2014)(Nakashima & Iohara 2014)(Mayo et al. 2014)(Albuquerque et al. 2014)(Huang & Garcia-Godoy 2014).

Ainsi, les objectifs que nous avons définis pour notre travail sont les suivants :

- Réaliser une revue bibliographique décrivant les principales conditions de culture actuellement utilisées pour obtenir des cellules à partir de la pulpe dentaire humaine. Ce travail a permis de mettre en exergue certains points qui peuvent s'avérer limitants dans la perspective de produire des CPD comme un MTI et selon les BPF **(Article 2)**.

- Améliorer la qualité des CPD obtenues *ex vivo* avec une approche de respect de l'intégrité cellulaire et de sécurité pour le patient receveur. Pour cela, nous avons décidé de mettre au point un protocole global permettant la production de CPD en respectant le plus possible les BPF **(Article 3).** Nous avons ensuite comparé, à l'aide de marqueurs membranaires, les CPD avec les cellules mésenchymateuses de la gelée de Wharton isolées et amplifiées de manière similaire **(Article 4).** Un travail de comparaison plus large a également été réalisé avec des cellules mésenchymateuses de moelle osseuse et de tissu adipeux **(Article en préparation).**

RESULTATS

Article 2 : Fabrication d'un produit cellulaire à partir de la pulpe des troisièmes molaires : stratégies actuelles et perspectives de recherche

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Manufacturing of dental pulp cell-based products from human third molars: current strategies and future investigations.

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Résumé : Ces dernières années, des thérapies à base de cellules mésenchymateuses souches/progénitrices ont été développées pour améliorer les stratégies de réparation des tissus humains. Dans ce contexte, la dent est récemment apparue comme une source intéressante de cellules souches ou progénitrices pour régénérer les tissus oro-faciaux. En effet, la pulpe dentaire est facile d'accès et les cellules mésenchymateuses pulpaires présentent un fort potentiel de différenciation. Les différents organismes de contrôle recommandent actuellement d'utiliser des procédures standardisées pour l'isolement, le stockage et l'expansion des cellules en culture afin de garantir une sécurité, une efficacité et une reproductibilité optimale lorsque les cellules sont utilisées pour une application clinique. Cependant, la plupart des procédures utilisées pour la production de cellules à partir de la pulpe dentaire ne sont pas entièrement satisfaisantes car elles peuvent altérer les propriétés biologiques des cellules mais aussi la qualité des produits qui en sont composés. Les procédures d'isolement, d'enrichissement, de cryopréservation et d'amplification cellulaire pendant de nombreux passages et dans des milieux contenant des produits d'origine animale ou humaine sont connues pour modifier le phénotype, la viabilité, la prolifération et les capacités de différenciation des cellules. Cet article se focalise sur les stratégies actuelles de fabrication des produits médicinaux cellulaires à partir de la pulpe dentaire et propose un nouveau protocole pour améliorer l'efficacité, la reproductibilité et la sécurité des stratégies thérapeutiques en cours de développement.

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Manufacturing of dental pulp cell-based products from human third molars: current strategies and future investigations

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In recent years, mesenchymal cell-based products have been developed to improve surgical therapies aimed at repairing human tissues. In this context, the tooth has recently emerged as a valuable source of stem/progenitor cells for regenerating orofacial tissues, with easy access to pulp tissue and high differentiation potential of dental pulp mesenchymal cells. International guidelines now recommend the use of standardized procedures for cell isolation, storage and expansion in culture to ensure optimal reproducibility, efficacy and safety when cells are used for clinical application. However, most dental pulp cell-based medicinal products manufacturing procedures may not be fully satisfactory since they could alter the cells biological properties and the quality of derived products. Cell isolation, enrichment and cryopreservation procedures combined to long-term expansion in culture media containing xeno- and allogeneic components are known to affect cell phenotype, viability, proliferation and differentiation capacities. This article focuses on current manufacturing strategies of dental pulp cell-based medicinal products and proposes a new protocol to improve efficiency, reproducibility and safety of these strategies.

Keywords: human dental pulp, stem cells, tissue engineering, immunophenotyping, expansion, cryopreservation, good manufacturing practices, cell-based medicinal products

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Introduction

Over the two last decades, mesenchymal stromal cells (MSC) have been intensely studied due to their potential clinical applicability to treat tissue and organ defects resulting from diseases, trauma or aging (Caplan, 1991). Their use has been proposed to repair and regenerate human mesenchymal tissues, alone or combined to scaffolds and/or morphogenic molecules (Langer and Vacanti, 1993). Bone marrow and adipose tissue are conventional sources of MSC, but invasive cell collection protocols, frequent use of general anesthesia and risk of morbidity at the collection site have stimulated the search for alternative tissues (Huang et al., 2009; Zuk, 2010; Davies et al., 2014). Third molars are frequently removed for therapeutic reasons and the connective tissue it

contains, the dental pulp, can be easily recovered. They are now considered a valuable source of MSC for tissue repair and regeneration (Mayo et al., 2014). In this context, numerous investigators have attempted to obtain clinical-grade dental pulp stem/progenitor cells (DPSC) from these teeth. However, most manufacturing procedures reported so far may not be totally satisfactory, since they may alter the biological properties of the cells and the quality of the derived cell-based products (Ménard and Tarte, 2013). If such procedures are currently permitted by European and American regulation authorities, further studies are necessary to develop more efficient, reproducible, safe and standardized manufacturing processes of dental pulp cell-based medicinal products (Tirino and Papaccio, 2012; Albuquerque et al., 2014; Eubanks et al., 2014; Huang and Garcia-Godoy, 2014; La Noce et al., 2014; Nakashima and Iohara, 2014).

Dental pulp mesenchymal cells have been successfully used to regenerate human craniofacial bone (d'Aquino et al., 2009; Giuliani et al., 2013). However, these studies were performed in the absence of defined, universally accepted protocols for large-scale, clinical-grade production of DPSC (Fekete et al., 2012). This point is important in the light of recent reports indicating moderate, irreproducible and non-suitable benefits of therapies performed with various sources of MSC (Allison, 2009; Tyndall, 2011; Daley, 2012). These results were explained in part by the fact that cell performances are affected by cell isolation and expansion conditions and indicate the need for optimized and standardized procedures for MSC-based products' manufacturing (Allison, 2009; Pacini, 2014). The European Union (EU) and United States (US) have established classifications and recommended guidelines for manufacturing MSC-based products. In Europe, MSC are defined as "cell therapy products" and referred to as Advanced Therapy Medicinal Products (ATMP) (European Regulation 1394/2007). ATMP are considered Cell-Based Medicinal Products (CBMP) when containing living cells or tissues. CBMP are "medicinal products presented as having properties for, or used in or administered to, human beings with a view to treating, preventing or diagnosing a disease in which the pharmacological, immunological or metabolic actions are carried out by cells or tissues" (Schneider et al., 2010; Pacini, 2014). DPSC belong to this category and can be referred to as Dental Pulp (DP)-CBMP. In the US, DPSC are considered as Human Cells, Tissues or cellular and tissue-based Products (HCT/Ps) (Code of Federal Regulation (CFR) Title 21 CFR 1271). They are classified in two categories: (1) products that are "minimally manipulated" and used clinically in a homologous manner, and (2) products that are either "more than minimally manipulated" or used in a non-homologous manner. A cell-based product is considered as being "more than minimally manipulated" when the inherent biological characteristics of the cells have been significantly altered (Pacini, 2014).

Production and delivery of MSCs should be made in accordance with European Good Manufacturing Practices (GMP), whereas, in the US, it must comply with Current Good Tissue Practice requirements (GTP) (Fekete et al., 2012; Kellathur and Lou, 2012; Sensebé et al., 2013). GMP/GTP require many quality controls regarding donor eligibility, sample recovery, label, transport and receipt, process and storage, laboratory equipment, supplies and reagents, cell-based product distribution to recipient patients and documentation that must be maintained by the handler (Alici and Blomberg, 2010; Abou-El-Enein et al., 2013; Sensebé et al., 2013; Wuchter et al., 2015). These controls make GMP/GTP procedures long and costly, and further studies are encouraged to develop shorter, less expensive and more standardized procedures for DP-CBMP manufacturing (Albuquerque et al., 2014; Eubanks et al., 2014; Huang and Garcia-Godoy, 2014; La Noce et al., 2014; Nakashima and Iohara, 2014; Hilkens et al., 2015). In the present paper, we will firstly review current international guidelines regarding the five manufacturing steps of DP-CBMP (**Figure 1**), and then we will highlight the drawbacks and potential risks of actual strategies. Finally we will propose modifications of the protocols intended to increase the efficiency, reproducibility and safety of these strategies, from tooth extraction to the harvest of clinical-grade DP-CMBP.

Teeth Collection and Pulp Tissue Recovery

Since the discovery of DPSC by Gronthos et al. (2000), numerous papers have reported the isolation of stem/progenitor cells from the dental pulp of human third molars. However, since there are no rules specifying the best tooth development stages for pulp cell collection, teeth were collected in patients of various ages and therefore at various developmental stages. It also greatly impairs the interpretation and comparison of the experimental results.

Transport from the operating block to the laboratory notably requires a medium that does not affect cell viability. It was previously shown that DPSC remain viable up for 5 days when extracted teeth are maintained in phosphate-buffered saline (PBS) (Perry et al., 2008; Woods et al., 2009) and this time is more than enough for the transport of samples to the laboratory and pulp recovery.

Dental Pulp Cell Isolation and Enrichment

After dental pulp recovery, two options are possible for isolating dental pulp cells: enzymatic dissociation and explant culture. Enzymatic dissociation consists of digesting the pulp tissue with collagenase and dispase enzymes to liberate the cells that are then plated on culture dishes. However, a growing number of authors consider that enzymatic dissociation is not adapted to medicinal manufacturing, owing to its putative consequences on cell phenotype and properties (Shah et al., 2013; Busser et al., 2014; Ohnuma et al., 2014). In addition, tissues and cells exposed to collagenase are considered "more than minimally manipulated" by FDA [Code of Federal Regulation (CFR) Title 21 CFR 1271] and potentially require the use of pharmaceutical grade manufactured enzymes, which significantly increases the scale-up costs. By contrast, cell isolation by explant culture increasingly appears easier, faster, safer, less expensive and more in line with GMP guidelines to obtain clinical-grade amounts of MSC (Hilkens et al., 2013). It is based on the growth of cells out of tissue fragments (explants) that are plated on culture dishes. It recently allowed for efficient recovery of human adipose or Wharton jelly stem/progenitor cells in serum-free,

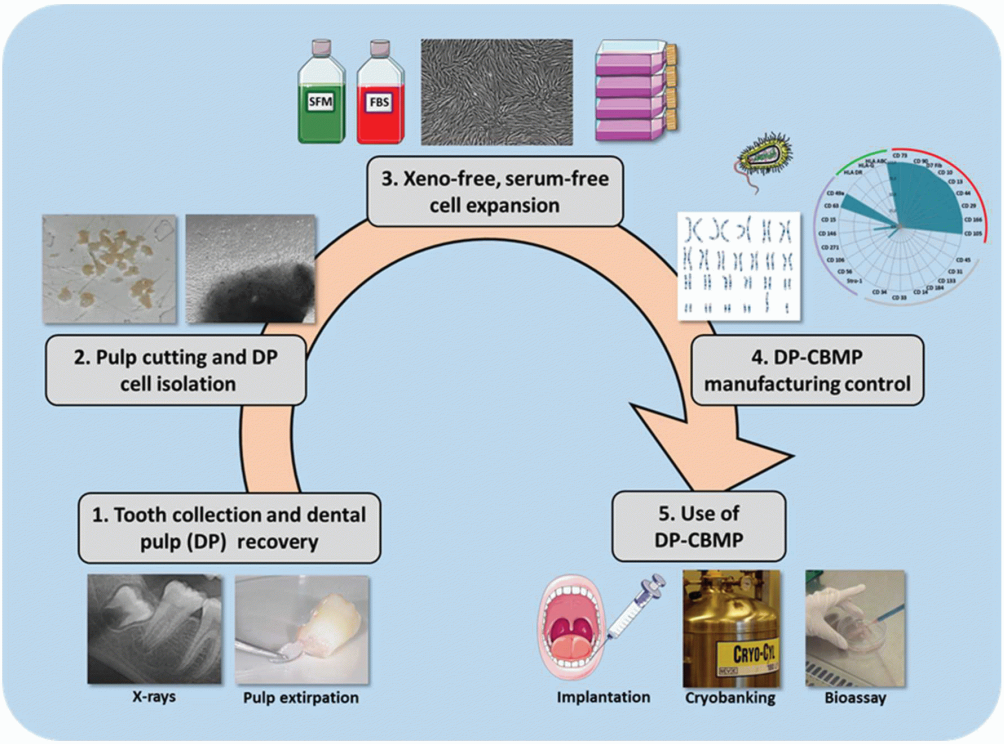

FIGURE 1 | Standardization of the DP-CBMP manufacturing process. It requires five major steps: tooth selection and use of the easiest technique for pulp recovery (Step 1), HDPC isolation with fast, safe and less expensive procedures (Step 2), cell expansion in defined, serum-free culture conditions with xeno-free reagents (Step 3), advanced monitoring and control of DP-CBMP manufacturing (Step 4), use of clinical-grade DP-CMBP, for immediate implantation, cryobanking or development of a bioassay (Step 5).

xeno-free medium conditions (Busser et al., 2014; Swamynathan et al., 2014). Additionally, explant-derived DPSC display similar or enhanced differentiation abilities compared with cells from dissociated tissue (Spath et al., 2010; Hilkens et al., 2013).

Cell selection by sorting methods has been proposed to enrich the cultured cell population in stem/progenitor cells based on their expression of specific surface markers such as CD34, CD184, Stro-1, CD146, CD271, and MSCA-1 (Shi and Gronthos, 2003; d'Aquino et al., 2009; Waddington et al., 2009; Yu et al., 2010; Jiang et al., 2012; Tomlinson et al., 2015). However, the use of such a procedure is today limited by the complexity of the technique and the prohibitive cost (Kawashima, 2012; Nakashima and Iohara, 2014). Besides, multiplying steps and using additional reagents increase the risk of microbial contamination and the difficulty to obtain CBMP in GMP conditions. The same reservation can be made regarding the use of biophysical markers that have been found relevant to isolate MSC in an easier and more predictable way than biochemical markers (Lee et al., 2014).

Dental Pulp Cell Culture and Expansion

Among other factors, the composition of the culture medium and the presence of a coating material on the culture dish may influence the nature and the quality of the final CBMP and therefore the clinical results (Lopez-Cazaux et al., 2006; Majd et al., 2009; Jung et al., 2012; Pisciotta et al., 2012; Pacini et al., 2014). Currently, CBMP manufacturing under GMP procedures recommends the use of xeno-free materials and reagents to prevent the risk of viral, bacterial, fungal and prion contamination, and the possible induction of immunizing effects in the final recipient. Additionally, industrial production is responsible for frequent batch-to-batch serum variability and the serum itself can promote early cell differentiation (Mannello and Tonti, 2007; Jung et al., 2012). For these reasons, the supplementation of the cell culture medium with xeno- or allogeneic products should be limited to "cases for which a valid alternative cannot be found" (European Regulation 1394/2007). Today, the development of xeno-free, serum-free, defined media, able to rapidly expand stem/progenitor cells without impairing their differentiation capabilities, represents a major objective for the standardization of DP-CBMP production (Tekkatte et al., 2011; Jung et al., 2012; Bonnamain et al., 2013; Carvalho et al., 2013). Multiple passages are often necessary to obtain a clinical-scale amount of cells, but they may lead to a slow-down of the proliferation rate, progressive cell senescence and loss of multipotentiality that prevent

future cell differentiation (Baxter et al., 2004; Bork et al., 2010; Yu et al., 2010; Sensebé et al., 2013). In our culture conditions, cell doubling times remained constant from P1 to P4 (≈40 h) and we calculated that more than 25.10^7 cells could be theoretically obtained after four passages with one dental pulp, which is likely to be a sufficient cell number for one pulp regeneration, bone socket filling, or for localized periodontal treatment (Kaigler et al., 2013; Albuquerque et al., 2014).

DP-CBMP Manufacturing Controls

DP-CBMP manufacturing requires advanced quality controls of the safety, identity and efficacy of the final product (Wang et al., 2005; Sensebé et al., 2013). Since CBMP cannot undergo sterilization before implantation, the absence of bacteria, virus, fungi and prion contamination has to be checked. The presence of endotoxin must also be tested to prevent immune reactions in the recipient patient. Long-term *ex vivo* expansion of cells increases the risk of genetic instability and the occurrence of potential chromosomal abnormalities, since there exists a close relation between cell senescence and risk of transformation (Baxter et al., 2004; Rubio et al., 2005; Campisi, 2007). To limit this risk, the number of population doublings should be kept to a minimum. In addition, conventional karyotyping must be combined with fluorescence *in situ* hybridization (FISH) or comparative genomic hybridization (CGH array) to assess the genomic stability of scaled-up cell populations (Barkholt et al., 2013).

The control of the population identity into expanding cell cultures is generally realized by flow cytometry analysis of surface antigens. During the past decade, most of these controls have been realized in compliance with the recommendations of the International Society of Cellular Therapy (ISCT) (Dominici et al., 2006). However, it is today acknowledged that several markers initially proposed by ISCT for the positive characterization of MSC (for instance CD73, CD90, and CD105) are shared by several populations of cells including progenitor cells, mature fibroblasts or perivascular cells (Russell et al., 2010; Alt et al., 2011; Halfon et al., 2011; Al-Nbaheen et al., 2013; Lv et al., 2014).

DP-CBMP Uses

Over recent years, DP-CBMP were clinically tested with the aim to regenerate human craniofacial bone. DP-CBMP were implanted, in association with a collagen I-based sponge scaffold, in mandibular bone sockets in a phase I clinical trial (d'Aquino et al., 2009). Three years after DP-CBMP grafting, the tissue regenerated in the graft site was compact bone (Giuliani et al., 2013). Case reports of osteoradionecrosis treatment using DP-CBMP were also reported (Manimaran et al., 2014). The angiogenic, neurogenic and odontogenic potential of DP-CBMP was also successfully tested in preclinical studies (Gandia et al., 2008; Iohara et al., 2009; Sakai et al., 2012; Ishizaka et al., 2013). In addition, a phase I clinical trial is currently under progress to evaluate the DP-CBMP potential to regenerate the human dental pulp (Nakashima and Iohara, 2014). Despite these successes, potential applicability of DP-CBMP will be closely dependent on their final production cost and their large-scale clinical outcomes. In particular, a high cost-efficacy ratio would constitute a serious impediment for their routine use. Hence, it is necessary to have a clear overview and understanding of the complete value chain to try to reduce costs (Abou-El-Enein et al., 2013, 2014; Leijten et al., 2014).

Storage of cryopreserved cell-based products (cryobanking) over long periods of time offers unique opportunities to increase DP-CBMP applicability. However, similar to cell culture and expansion, cryopreservation is associated with infective, prion, toxicological and immunological risks owing to the presence of human or animal components and additives such as DMSO in the storage medium (Papaccio et al., 2006; Perry et al., 2008; Woods et al., 2009; Lee et al., 2012). Accordingly, xeno-free, defined cryopreservation media must be privileged.

DP-CBMP could also be used in biomedical research as components of bioassay kits to investigate the effects of drugs on dental pulp cells in a reproducible "humanized" system (Jurga et al., 2010; Leeb et al., 2011; Forraz et al., 2013). Such kits are reliable preclinical alternatives to animal models in the actual regulatory context. Assessment of the risks related to chemical products' use and screening or testing new therapeutic molecules are indeed extremely complicated and costly. The average costs to take a blockbuster drug to clinical trials are estimated to be around 1 billion euros. Furthermore, the accuracy of toxicological and preclinical studies greatly depends on the experimental animal models used for such evaluations. In particular, rodent species, widely use, are known to only partially mimic the human biological system. Development of DP-CBMP bioassay kits would offer a prime platform to successfully induce dentinogenesis, osteogenesis or neurogenesis *in vitro* (Zhang et al., 2006; Leijten et al., 2014; Woloszyk et al., 2014; Jensen et al., 2015).

Proposals for a Protocol with a More GMP Compliant Approach (Figure 2)

We recently proposed the use of impacted third molars between Nolla's developmental stages 5 (crown almost completed) and 7 (one third root completed). The presence of large, open apices in teeth without roots or with roots partially developed allows for an easy access to the pulp tissue and its gentle, atraumatic extirpation from the enamel/dentin shell with fine tweezers. It avoids the cell stress resulting from the crown-root mechanical separation with a drill or a clamp that is necessary for recovering pulps from teeth with more developed or complete roots (Perry et al., 2008; Takeda et al., 2008; Ducret et al., 2015). Additionally, human dental pulp cells (HDPC) isolated at around the crown-completed stage displayed short cell doubling times and high growth rate (Takeda et al., 2008). We found similar results in our study. We also selected impacted teeth to minimize the risk of pulp tissue contamination and disease transmission by oral microorganisms (Nolla, 1960; Ducret et al., 2015). This choice may enable to skip the step of sample disinfection performed

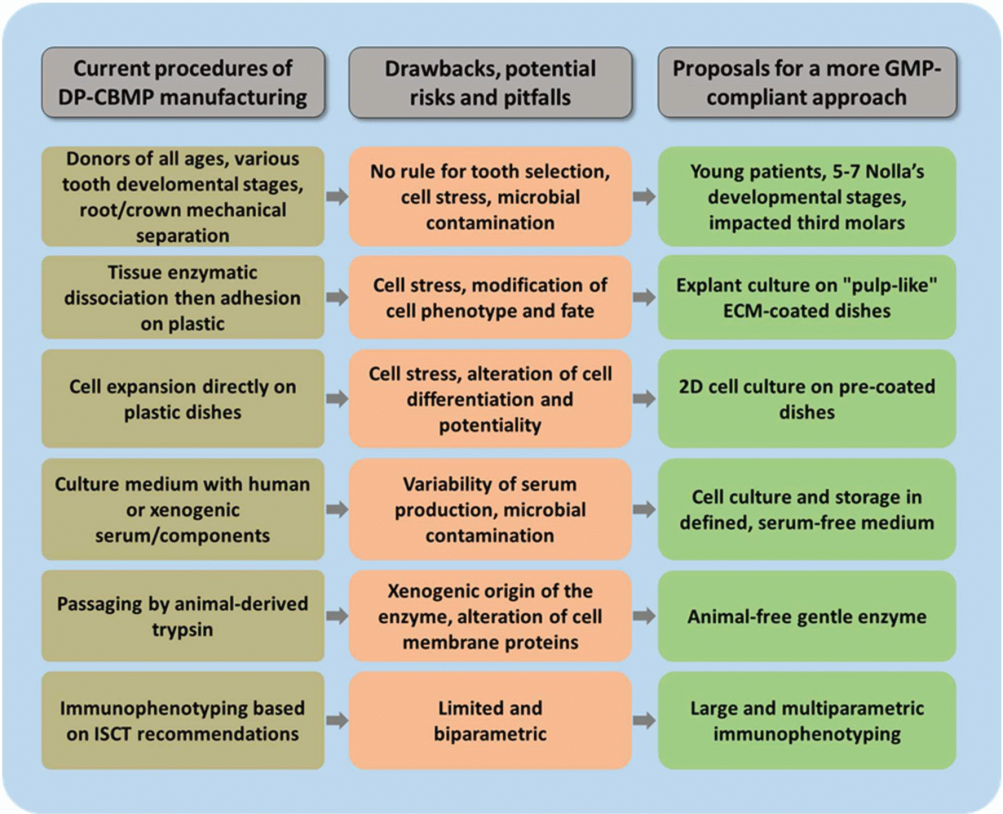

FIGURE 2 | Important steps during DP-CBMP manufacturing that require modification. Major drawbacks, risks and pitfalls are highlighted for each one, and proposals are made for the development of more GMP-compliant procedures.

with chemicals such as chlorhexidine or povidone-iodine/sodium thiosulfate (Perry et al., 2008; d'Aquino et al., 2009). When using PBS as a transport medium, we failed to detect any contamination in cultures of HDPC ($n > 50$ patients) during the isolation and expansion steps, contrary to others (Perry et al., 2008; Ducret et al., 2015). This might be related to our selection of impacted teeth from young patients (13–17 year-old) that have never been in contact with the septic oral cavity, versus the selection of erupted ones from older patients (18–30 year-old) by those authors.

Regarding cell isolation, we used explant culture for recovering human dental pulp cells for clinical application. Each pulp sample was cut into about 20 explants that allowed for the harvest of a total of one million dental pulp cells after 14 days of culture (Ducret et al., 2015). This result is in agreement with other studies reporting that, whatever the technique used (tissue dissociation or explant culture), 2 weeks of culture allow for the recovery of about 10^6 cells from one third molar pulp (Eubanks et al., 2014).

We pre-coated the culture dish surface for cell isolation and culture with an equal mixture of human placental collagens I and III. This composition was chosen because they are the two most abundant collagens in the dental pulp extracellular matrix. Xeno-free dissociating reagents (such as TrypLe® or Accutase®) and xeno-free defined culture medium (such as SPE-IV® [ABCell-Bio, France], containing clinical grade human albumin, α-MEM, rhIGF-1 and rhFGF-2) are recommended for cell culture and passaging instead of the products commonly used (Carvalho et al., 2013; Ducret et al., 2015). Moreover, cryopreservation of dental pulp cells in serum-free medium had no negative impact on cell doubling times and cumulative cell numbers (Ducret et al., 2015). Although the viability of cells cryopreserved in serum-free medium was decreased compared to fresh cells, it is similar to that previously reported (Lee et al., 2012).

Future investigations are required for identifying more specific membrane markers for these cells. In our study, immunophenotypic analysis of 17 surface markers revealed that our dental pulp cell expanding population was made of mesenchymal cells, a percentage of whom expressed the mesenchymal stem cell/progenitor markers CD146 and MSCA-1. The number of cells expressing these markers remained similar from P1 to P4, suggesting that the cell fate was not significantly

affected by our culture conditions. In addition, cell karyotyping by G-band analysis showed that this rapid expansion did not lead to genomic instability that would be potentially harmful for the recipient patient (Ducret et al., in press).

Conclusion and Perspectives

Recent successes in bone and dental pulp regeneration therapies carry the promise to use dental pulp-cell-based medicinal products in the near future. However, current strategies to manufacture DP-CBMP are not totally satisfactory since they do not comply with current international guidelines. New manufacturing standardized protocols, intended to increase efficiency, reproducibility and safety of these strategies, are urgently needed. Further investigations are also warranted to estimate the real benefit of DP-CBMP use compared to current therapeutic options and precisely determine the cost-efficacy ratio that risks being a major block for the large-scale clinical use of these cell-based products.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Synthèse et conclusions de l'article 2 :

Les produits cellulaires destinés à l'ingénierie tissulaire sont référencés comme « médicaments de thérapie innovante » et les lignes directrices des autorités européennes et américaines (notamment la Directive Européenne 1394/2007 et le 21ème Code américain de Régulation Fédérale Partie 1271) ont précisé des conditions des Bonnes Pratiques de Fabrication pour ces produits cellulaires. Ces textes incluent en particulier des recommandations concernant les protocoles de culture cellulaire afin d'assurer une reproductibilité, une efficacité et une sécurité optimales du produit cellulaire final destiné à être introduit chez le patient. Ces protocoles doivent altérer le moins possible les caractéristiques biologiques des cellules et éviter tout risque pour le patient.

Dans ce contexte, la très grande majorité des protocoles rapportés dans la littérature ne sont pas satisfaisants. En effet, ils comprennent le plus souvent une amplification importante des cellules avec de nombreux passages qui peuvent entrainer une sénescence importante des cellules et une modification de leur phénotype. De même, les milieux utilisés pour la culture cellulaire contiennent la plupart du temps des éléments xénogéniques ou allogéniques qui peuvent être contaminés par des virus, des bactéries ou le prion.

L'objectif de ce premier travail était de réaliser une large analyse de la bibliographie afin de synthétiser les principales conditions d'obtention des CPD. Les résultats de cette analyse nous ont permis de proposer cinq étapes lors de la production de CPD : collecte des dents et récupération de la pulpe dentaire, isolement des cellules et enrichissement, culture et expansion des cellules, contrôle de la production, et utilisation des cellules (Figure 25). Ce travail a également permis de souligner un certain nombre de facteurs qui peuvent s'avérer limitants dans la perspective de produire des CPD comme un MTI et selon les BPF. Dans la dernière partie de l'article, nous proposons une modification des protocoles d'isolement et de culture cellulaire classiquement décrits tenant compte de ces facteurs (Figure 26).

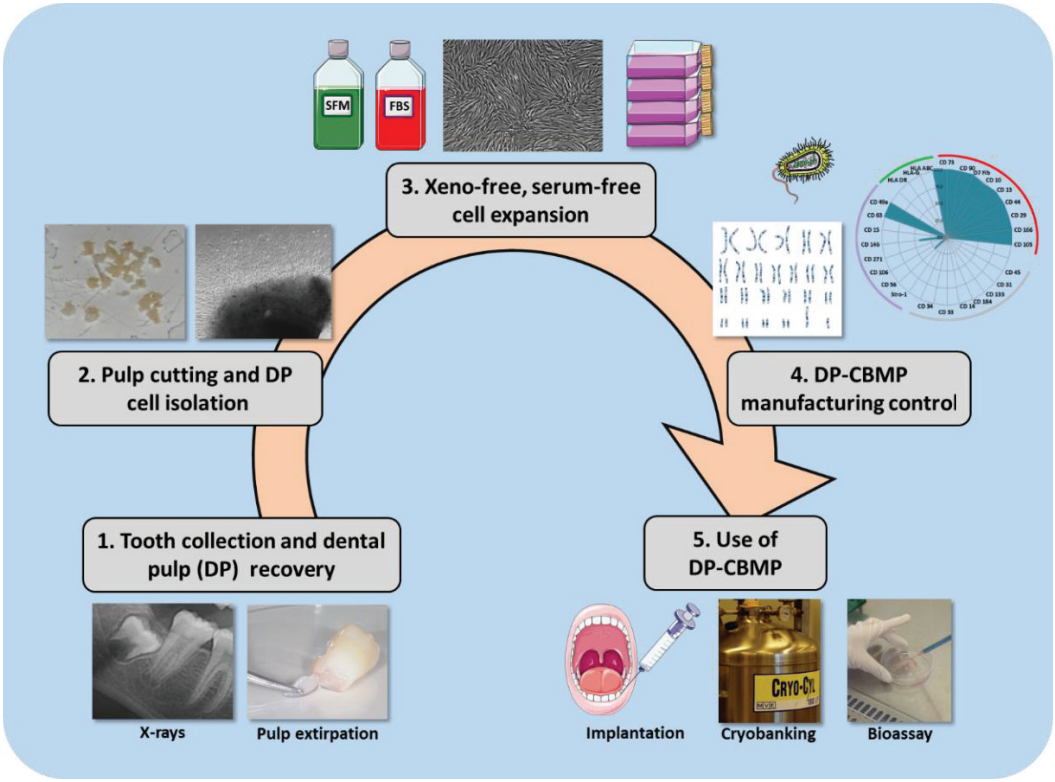

Figure 25 : Les 5 étapes de fabrication d'un produit cellulaire composé de cellules pulpaires.

Current procedures of DP-CBMP manufacturing	Drawbacks, potential risks and pitfalls	Proposals for a more GMP-compliant approach
Donors of all ages, various tooth develomental stages, root/crown mechanical separation	No rule for tooth selection, cell stress, microbial contamination	Young patients, 5-7 Nolla's developmental stages, impacted third molars
Tissue enzymatic dissociation then adhesion on plastic	Cell stress, modification of cell phenotype and fate	Explant culture on "pulp-like" ECM-coated dishes
Cell expansion directly on plastic dishes	Cell stress, alteration of cell differentiation and potentiality	2D cell culture on pre-coated dishes
Culture medium with human or xenogenic serum/components	Variability of serum production, microbial contamination	Cell culture and storage in defined, serum-free medium
Passaging by animal-derived trypsin	Xenogenic origin of the enzyme, alteration of cell membrane proteins	Animal-free gentle enzyme
Immunophenotyping based on ISCT recommendations	Limited and biparametric	Large and multiparametric immunophenotyping

Figure 26 : Procédures actuelles de fabrication d'un produit cellulaire à partir de la pulpe dentaire qui nécessitent des modifications.

L’utilisation récente de CPD pour un essai clinique de stade 1 dont le recrutement des patients vient de se terminer est encourageant dans la perspective de futures applications thérapeutiques à grand échelle (Essai numéro : UMIN000009441)(Nakashima & Iohara 2014). Cependant, il reste encore de nombreuses améliorations à réaliser, comme notamment trouver une alternative au G-CSF, le facteur de croissance utilisé dans cette étude, dont les effets sur les cellules et les tissus sont encore mal connus. Le design de cette étude et la production des cellules pourront aussi être améliorés pour s’assurer de la sécurité, de l’efficacité et de la reproductibilité du produit cellulaire fabriqué. Dans ce contexte, le développement de nouveaux protocoles pour la production de CPD selon des procédures de BPF apparait nécessaire (Article 3 et Article 4).

Article 3 : Production de cellules de la pulpe dentaire selon une approche médicinale

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Production of human dental pulp cells with a medicinal manufacturing approach.

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Introduction : Les cellules de la pulpe dentaire CPD sont généralement isolées et cultivées avec des produits xénogéniques et dans des conditions de stress qui peuvent altérer leurs caractéristiques biologiques. Cependant, les recommandations de l'Agence américaine pour la nourriture et les médicaments (FDA) et l'agence européenne des médicaments (EMA) proposent actuellement d'utiliser des protocoles de production comparables à ceux utilisés pour la fabrication des médicaments. Le but de notre travail a donc été de développer un protocole permettant de produire *ex vivo* de grandes quantités de CPD pour l'ingénierie de la pulpe dentaire et de l'os, selon ces recommandations internationales. ***Méthodologie*** : Les CPD humaines ont été obtenues à partir de cultures d'explant. Après sélection du milieu sans sérum approprié, les CPD en culture ont été analysées par immunophénotypage par cytométrie de flux. Les échantillons ont ensuite été cryopréservés pendant 510 jours. Après décongélation, le temps de doublement cellulaire a été déterminé jusqu'au passage 4. L'analyse du caryotype a été réalisée par analyse des bandes G. Le potentiel de différentiation osteo/odontoblastique a été déterminé après culture des CPD dans un milieu de différenciation par analyse de l'expression de gènes de marqueurs ostéo/odontoblastiques et par la quantification de la minéralisation. ***Résultats*** : L'immunophénotypage des CPD en culture a révélé un profil mésenchymateux des cellules dont certaines exprimaient les marqueurs de cellules souches/progénitrices CD271, Stro-1, CD146 ou MSCA-1. Le temps de doublement des CPD après décongélation était stable et similaire à celui des cellules fraichement isolées. Apres amplification, les cellules ne présentaient pas d'anomalies du caryotype. L'expression des gènes codant pour la phosphatase alcaline, l'ostéocalcine et la sialophosphoprotéine dentinaire, ainsi que la minéralisation des cultures étaient augmentées lorsque les cellules étaient cultivées dans un milieu de différenciation ostéo/odontogénique. ***Conclusions*** : Nous avons réussi à isoler, cryopréserver et amplifier des CPD humaines avec une approche de fabrication médicinale. Ces travaux peuvent constituer une base pour déterminer comment la production de CPD peut être optimisée pour l'ingénierie de la pulpe dentaire ou de l'os.

Production of Human Dental Pulp Cells with a Medicinal Manufacturing Approach

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Abstract

Introduction: Human dental pulp cells (HDPCs) are generally isolated and cultured with xenogeneic products and in stress conditions that may alter their biological features. However, guidelines from the American Food and Drug Administration and the European Medicines Agency currently recommend the use of protocols compliant with medicinal manufacturing. Our aim was to design an *ex vivo* procedure to produce large amounts of HDPCs for dentin/pulp and bone engineering according to these international recommendations. **Methods:** HDPC isolation was performed from pulp explant cultures. After appropriate serum-free medium selection, cultured HDPCs were immunophenotyped with flow cytometry. Samples were then cryopreserved for 510 days. The post-thaw cell doubling time was determined up to passage 4 (P4). Karyotyping was performed by G-band analysis. Osteo/odontoblastic differentiation capability was determined after culture in a differentiation medium by gene expression analysis of osteo/odontoblast markers and mineralization quantification. **Results:** Immunophenotyping of cultured HDPCs revealed a mesenchymal profile of the cells, some of which also expressed the stem/progenitor cell markers CD271, Stro-1, CD146, or MSCA-1. The post-thaw cell doubling times were stable and similar to fresh HDPCs. Cells displayed no karyotype abnormality. Alkaline phosphatase, osteocalcin, and dentin sialophosphoprotein gene expression and culture mineralization were increased in post-thaw HDPC cultures performed in differentiation medium compared with cultures in control medium. **Conclusions:** We successfully isolated, cryopreserved, and amplified human dental pulp cells with a medicinal manufacturing approach. These findings may constitute a basis on which to investigate how HDPC production can be optimized for human pulp/dentin and bone tissue engineering. *(J Endod 2015;■ :1–8)*

Key Words

Cryopreservation, human dental pulp, immunophenotyping, MSCA-1, osteo/odontoblast differentiation, tissue engineering

Dental research currently explores the potential of cell-based products and tissue engineering protocols to be used as alternatives to usual pulp/dentin and bone therapies. In this context, stem/progenitor cells appear to be particularly appropriate because of their high expansion ability and differentiation potential both *in vitro* and *in vivo* (1). If bone marrow and adipose tissue are considered potential sources of stem/progenitor cells, painful collection protocols, the decline of the amount of stem/progenitor cells with age, the necessity of general anesthesia, reduced proliferation capacity, and risk of morbidity at the collection site encourage the search for alternative candidates (1, 2). Human impacted third molars are frequently removed for therapeutic reasons and the loose connective tissue they contain; the dental pulp appears to be a valuable source of stem/progenitor cells for pulp/dentin and bone engineering. Indeed, it contains various cell populations that exhibit osteo/odontoblastic differentiation capabilities and that can be cryopreserved for periods of time greater than 6 months (3–5). Interestingly, human dental pulp cell (HDPC) populations were recently successfully used for regenerating human pulp/dentin and bone (6, 7).

Cell-based products for tissue engineering are now referred to as human cellular tissue-based products or advanced therapy medicinal products, and guidelines from the American Code of Federal Regulation of the Food and Drug Administration (21 CFR Part 1271) and the European Medicines Agency (European Directive 1394/2007) define requirements for appropriate cell production. These "good manufacturing practices" include recommendations regarding laboratory cell culture procedures to ensure optimal reproducibility, efficacy, and safety of the final medicinal product (8, 9). In particular, the Food and Drug Administration divides *ex vivo* cultured cells into "minimally" or "more than minimally" manipulated samples according to function of the use or not of procedures "that might alter the biological features of the cells." In this context, most if not all HDPC culture protocols that have been reported so far are unsatisfactory. Indeed, the use of xeno- or allogeneic cell culture media and long-term cell amplification are known to alter the quality of the final cell-based product (10–12). These findings make the design of new HDPC isolation, characterization, cryopreservation, and amplification *ex vivo* procedures that are compliant with good manufacturing practices regarding medicinal products necessary (5, 7, 13).

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The aim of this study was to define a protocol for obtaining a clinical scale number of HDPCs that possess osteo/odontoblastic differentiation potential. Therefore, HDPCs were grown from dental pulp explants on extracellular matrix–coated dishes, and their storage and amplification were performed in serum-free medium (SFM) by using xenogeneic-free products. After appropriate SFM selection, cell immunophenotype, viability, growth kinetics, karyotyping, and differentiation capacity were analyzed to validate our *ex vivo* protocol.

Materials and Methods

Isolation and Amplification of HDPCs

Healthy impacted human third molars were collected from donors aged 13–17 years with informed consent of the patients and their parents in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. Teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were used (14). Dental pulps were aseptically, gently extirpated from pulp cavities with fine tweezers, and the apical part of the radicular pulp was removed with a scalpel to prevent contamination by dental papilla cells. Pulps were then washed twice with phosphate-buffered saline containing 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Saint Aubin, France), referred to as P/S from herein, placed onto a sterile glass slide and cut with a scalpel into 0.5- to 2-mm^3 explants. The latter were cultured on dishes precoated with an equal mixture of human placental collagens I and III at a final concentration of 0.5 μg/cm^2 (ABCellBio, Paris, France). HDPCs outgrowing from the explants (referred to as passage 0[P0]-HDPCs) were detached from the culture dish after 2 weeks of culture with xeno-free recombinant protease TrypLe Select 1X (Life Technologies), counted, and either cryopreserved (see below) or plated (5×10^3 cells/cm^2) for amplification. The same protease was used for all subsequent passages.

Isolation Success Rate, Cell Outgrowth Surface Area, and Metabolic Assays

Three SFMs called SFM-1, SFM-2, and SFM-3 were tested for assessing HDPC outgrowth formation from the explants. Ninety pulp explants from 5 donors were pooled together to reduce interpatient variability and then separated into 3 equal groups to be cultured in SFM-1, SFM-2, or SFM-3. Explants were then seeded on collagen precoated 12-well plates and cultured in SFM-1 (composition: SPE-IV/EBM [a medium containing clinical grade human albumin, α-minimum essential medium (α-MEM), 25 ng/mL rhIGF-1, and 0.33 ng/mL rhFGF-2; ABCellBio] and P/S), SFM-2 (Dulbecco's Modified Eagle's Medium [DMEM]/F-12/Glutamax [Life Technologies], P/S, 5 ng/mL FGF-2 [R&D Systems, Lille, France] and 5 μg/mL insulin [Umuline, Lily, Neuilly-sur-Seine, France]) or SFM-3 (DMEM/F-12/Glutamax, 1% (v/w) Insulin/Transferrin/Selenium [Life Technologies], and P/S). Cultures were performed for 10 days and then fixed with 10% formalin and stained with a 0.5% crystal violet solution. The isolation success rate was defined as the percentage of explants that give rise to at least 1 HDPC outgrowth. The cell-covered surface area around each explant was calculated by using Image J software (National Institutes of Health, Bethesda, MD). For metabolic activity assessment, first-passage (P1) HDPCs from 5 different donors were pooled and then seeded (5×10^3/well) in collagen precoated 96-well plates. Cells were cultured for 68 hours in 200 μL SFM-1, SFM-2, or SFM-3, and then 40 μL of a 5-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added for 4 hours. Formazan product was solubilized with 99% dimethyl sulfoxide (Sigma-Aldrich) for 15 minutes, and metabolic activity was measured as absorbance at 590 nm with a Multiskan FC microplate reader (Thermo Fisher Scientific, Courtaboeuf, France).

Multicolor Flow Cytometry

Three million P1-HDPCs cultured in SFM-1 were stained with 17 fluorochrome-conjugated antibodies (Table 1). The nucleic acid dye 7-Amino-actinomycin D (7AAD; BD Biosciences, Le Pont de Claix, France) was used for the exclusion of nonviable cells. Samples were acquired on a BD FACSCanto II Flow cytometer (BD Biosciences) as uncompensated events and recorded as FCS 3.0 files. Analysis and compensation were performed using FlowJo vX software (FlowJo, Ashland, OR). The percentage of cells positively stained corresponded to the percentage of cells present within a gate established so that <1% of the measured positive events represented nonspecific binding by the fluorochrome-conjugated isotype-matched control. Additional fluorescence minus one controls were used for CD271, Stro-1, CD146, and MSCA-1.

Cryopreservation

HDPCs outgrowing from explants (P0-HDPCs) were detached with TrypLe Select 1X and counted. Cells were suspended (10^6/mL) in a solution containing 10% dimethyl sulfoxide and 90% cryogenic SFM (CRYO3; Stem Alpha, Saint-Genis l'Argentière, France). Cells were thoroughly mixed in cryotubes and immediately transferred to an isopropanol-filled Cryobox (Nalgene, Rochester, NY). Samples were then frozen in liquid nitrogen for 510 days.

Cell Viability, Cumulative Doubling Number, and Doubling Time Determination

Post-thaw P1-HDPC viability was compared with that of fresh P1-HDPCs. Cells were stained with trypan blue to discriminate between live and dead cells, and they were counted with a Cellometer auto T4 (Nexcelom Bioscience, Lawrence, MA). For cumulative doubling number (CDN) and doubling time (DT) analysis, HDPCs from P1 to P4 were plated (5×10^3 cells/cm^2) on T12.5 flasks (Corning Inc, Corning, NY) until they reached 80%–90% confluence. CDN and DT were calculated from the addition of doubling number counts and the time of culture according to the following formulae:

$$\mathrm{CDN} = \ln(\mathrm{nf}/\mathrm{ni})/\ln 2 \quad (1)$$

$$\mathrm{DT} = \mathrm{CT}/\text{doubling number}(\mathrm{nf} = \text{final number of cells at 80\% confluence, ni} = \text{initial number of cells, and CT} = \text{culture time}) \quad (2)$$

Karyotyping

Post-thaw P4-HDPCs were exposed for 3 hours to 0.7% colcemid (Life Technologies) diluted in the culture medium, and then cells were detached and centrifuged. The pellet was then resuspended in 0.075 mol/L KCl for 2 minutes at room temperature. Cells were centrifuged again, resuspended in methanol acetic acid (3:1) fixative, and stored at −20°C for at least 2 days. G-band staining was performed with the Leishman-Giemsa cocktail.

Osteo/odontoblastic Differentiation and Mineralization Quantification

Post-thaw HDPCs were plated (4.2×10^3 cells/cm^2) on plastic dishes and amplified. Once confluence was reached (3–4 days), cells

TABLE 1. Fluorochrome-conjugated Monoclonal Antibodies Used for Immunophenotypic Analysis

Marker	Isotype	Supplier	Reference	Fluorochrome
7AAD	NA	BD Biosciences	559925	Staining solution
CD73	Ms IgG1, κ	BD Biosciences	561258	PE-Cy7
CD10	Ms IgG1, κ	BD Biosciences	555375	PE
CD13	Ms IgG1, κ	BD Biosciences	557454	APC
CD29	Ms IgG1, κ	BD Biosciences	559883	APC
CD44	Ms IgG2b, κ	BD Biosciences	560532	APC-H7
CD49a	Ms IgG1, κ	BD Biosciences	559596	PE
CD90	Ms IgG1, κ	BD Biosciences	555595	FITC
CD105	Ms IgG1, κ	BD Biosciences	560839	PE
CD166	Ms IgG1, κ	BD Biosciences	559263	PE
CD14	Ms IgG2b, κ	BD Biosciences	560180	APC-H7
CD34	Ms IgG1, κ	BD Biosciences	555824	APC
CD45	Ms IgG1, κ	BD Biosciences	560777	V500
HLA-DR	Ms IgG2a, κ	BD Biosciences	561224	V500
CD271	Ms IgG1, κ	BD Biosciences	560834	PerCP-Cy5.5
Stro-1	Ms IgM, λ	Biolegend	340104	AF647
CD146	Ms IgG1, κ	BD Biosciences	560846	FITC
MSCA-1	Ms IgG1, κ	Biolegend	327308	APC

NA, not applicable.

were cultured for 4 weeks in a differentiation medium consisting of DMEM/F-12/Glutamax supplemented with 5% fetal calf serum (Life Technologies), P/S, 10^{-7} dexamethasone, 100 μmol/L ascorbate-2-phosphate, and 10 mmol/L β-glycerol phosphate (all from Sigma-Aldrich). Control cells were cultured in the amplification (control) medium.

For gene expression analysis with real-time polymerase chain reaction, total RNA was extracted by using the RNeasy Mini kit (Qiagen, Courtaboeuf, France). Reverse transcription (RT) was performed from 500 ng total RNA with Prime Script RT reagent kit (Takara; Ozyme, Montigny-le-Bretonneux, France) according to manufacturer's instructions. Real-time polymerase chain reaction amplifications were performed in a 20-μL reaction mix containing 10 μL Fast Start Universal SYBR green master (Roche, Mannheim, Germany), 4 μL RT template diluted 1:3 in sterile water, 300 nmol/L of each primer, and 4 μL sterile water. Amplification was performed in Rotorgene (Qiagen). Thermal cycling conditions consisted of an initial denaturation step at 95°C for 2 minutes and then 40 to 50 cycles of 95°C for 15 seconds and an annealing/extension step at 60°C for 30 seconds. Gene-specific primer sequences were as follows: osteocalcin (*OCN*), forward: 5′-GAAGCCCAGCGGTGCA-3′, reverse: 5′-CACTACCTCGCTGCCCTCC-3′; alkaline phosphatase, forward: 5′-AGCCCTTCACTGCCATCCTGT-3′, reverse: 5′-ATTCTCTCGTTCACCGCCCAC-3′; dentin sialophosphoprotein, forward: 5′-ATATTGAGGGCTGGAATGGGGA-3′, reverse: 5′- TTTGTGGC TCCAGCATTGTCA-3′; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), forward: 5′-ATGGGGAAGGTGAAGGTCG-3′, reverse: 5′-TAAAAGCAGCCCTGGTGACC-3′. The GAPDH housekeeping gene was used for sample normalization. Samples were performed in duplicate. The gene expression level was calculated as $2^{-\Delta Ct}$. The GAPDH Ct value was subtracted from the Ct value of the target gene, and results were expressed as relative gene expression to GADPH.

For the quantification of mineralization, HDPCs were fixed for 15 minutes in 10% formalin and then incubated in an alizarin red S (ARS) solution (pH = 4.1–4.3) (Sigma-Aldrich) for 20 minutes. ARS staining was visualized with a DM 750 phase-contrast microscope (Leica, Wetzlar, Germany) coupled to a color camera (ICC 50 HD; Nikon, Champigny-sur-Marne, France). Images were acquired with LAS EZ 2.0 software (Leica). Staining was quantified by removing ARS with a 10% acetic acid solution for 30 minutes, heating at 85°C for 10 minutes, centrifugation at 20,000*g* for 15 minutes, and neutralization with 10% ammonium hydroxide. Supernatants (150 μL) were dropped on a 96-well plate and read at 405 nm with a Multiskan FC microplate reader.

Statistical Analysis

Values were presented as mean ± standard deviation, and differences were analyzed using the Mann-Whitney *U* test for nonparametric analysis. The number of independent samples from different donors (*n*) is indicated in the figure legends. A P value <.05 was considered to be significant.

Results

Efficient isolation and expansion of HDPCs *ex vivo* require the use of an optimal culture medium. The isolation success rate, cell-covered dish surface area around the explant, and cell metabolic activity were determined for 3 different SFMs. After explant culture for 10 days, SFM-1 showed an outgrowth success rate 19.6% and 10% higher compared with SFM-2 and SFM-3, respectively (Fig. 1*A*). The cell outgrowth surface area around the explant increased in SFM-1 cultures by 2.78- and 5.64-fold compared with SFM-2 and SFM-3 cultures, respectively (Fig. 1*B*). HDPC metabolic activity was higher in SFM-1 than SFM-2 and SFM-3 (Fig. 1*C*). For these reasons, SFM-1 was selected for additional HDPC isolation and expansion experiments. Explant cultures performed with SFM-1 on collagen-coated dishes showed that the first HDPC started to grow from the explants after 3 to 5 days (Fig. 1*D*). Two weeks later, pooling of cells outgrowing from the explants from 1 dental pulp allowed harvesting of about 10^6 HDPCs (data not shown).

A multiparametric immunophenotypic analysis of cultured HDPCs was performed with a panel of 17 cell surface markers (Table 1). The gating tree was set so that the initial forward scatter/side scatter (FSC/SSC) gating represented the cell distribution based on size and intracellular composition. Then, the gate was set on 7AAD-negative, live cells (Fig. 2*A*). All HDPCs expressed the cell surface marker CD73, which was therefore chosen as a basis versus additional markers (Fig. 2*B*). Almost all CD73-positive HDPCs (≥97%) expressed the mesenchymal markers CD10, CD13, CD29, CD44, CD90, CD105, and CD166, whereas CD49a was expressed by a lower number of cells (85.3%) (Fig. 2*C*). The hematopoietic markers CD14 (monocyte marker), CD34 (early hematopoietic marker), CD45 (leukocyte marker), and human leukocyte antigen-DR (HLA-DR) (dendritic cell and macrophage marker) were expressed by very few cultured HDPCs

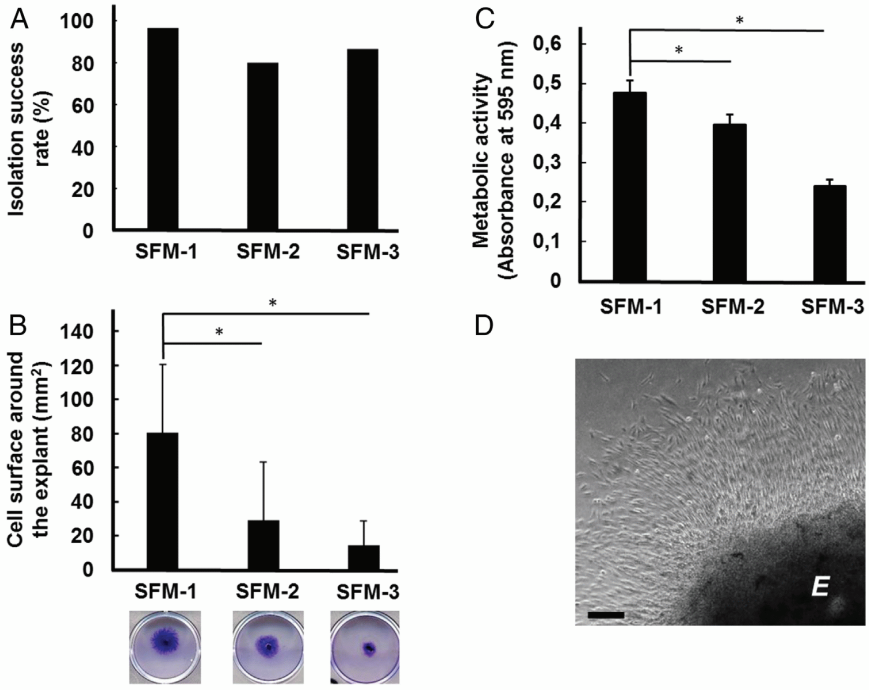

Figure 1. Selection of SFM for *ex vivo* culture of HDPCs. (*A*) SFM-1 (SPE-IV, insulin-like growth factor–1/fibroblast growth factor–2 [IGF-1/FGF-2]–containing medium) slightly increased the outgrowth success rate by 19.6% and 10% compared with SFM-2 (FGF-2/insulin-containing medium) and SFM-3 (insulin/transferrin/selenium-containing medium). (*B*) The cell surface area around the explant was 2.78- and 5.64-fold larger with SFM-1 compared with SFM-2 and SFM-3, respectively (n = 5). (*C*) HDPC metabolic activity was significantly increased in SFM-1 compared with other media (n = 7). (*D*) After 3 to 5 days of explant (E) culture in SFM-1, fibroblastlike HDPCs started to grow from the explant. *P < .05. Error bars: mean ± standard deviation. Scale bar = 100 μm.

(≤1%) (Fig. 2*D*). We then focused our immunophenotyping on the stem/progenitor cell markers CD271 (LNGFR), Stro-1, CD146 (MCAM), and MSCA-1. We observed that CD271 and Stro-1 were expressed by a very low number of cultured HDPCs (≤1%) but that CD146 and MSCA-1 were expressed by about 40% and 15% of cultured HDPCs, respectively (Fig. 2*E–H*). The expression level of stem/progenitor cell markers was similar from P1 to P4.

The viability of P1-HDPCs after 510 days of cryopreservation was compared with that of fresh P1-HDPCs. Results indicated that the viability of post-thaw HDPCs was significantly decreased compared with freshly isolated cells (Fig. 3*A*). However, this diminution had no significant impact on cell DTs that were stable and similar from P1 to P4 (≈40 hours) for both populations expanded in parallel (Fig. 3*B*). Cumulative doubling numbers after 4 passages were also similar (≈9) (Fig. 3*C*). The morphology of post-thaw and fresh HDPCs at P4 was fibroblastlike (Fig. 3*D*). The genomic stability of expanded post-thaw P4-HDPCs was shown by the observation of a normal karyotype upon G-band analysis (Fig. 3*E*). Mycoplasmas were not detected in random selected cultures of post-thaw cells using the MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME; data not shown).

The osteo/odontoblast differentiation potential of post-thaw HDPCs was confirmed by culturing cells in a specific differentiation medium containing dexamethasone, ascorbic acid, and β-glycerol phosphate. After 4 weeks of culture, the expression of genes coding for the osteo/odontoblastic proteins osteocalcin, alkaline phosphatase, and dentin sialophosphoprotein was significantly increased compared with cells maintained in the amplification (control) medium (Fig. 4*A*). In addition, ARS staining (Fig. 4*B*) and quantification (Fig. 4*C*) showed a higher mineralization degree in HDPC cultures maintained in the differentiation medium compared with cultures performed in the control medium.

Discussion

Current international guidelines refer to cell-based products derived from *ex vivo* amplified cells as human cellular tissue-based products or advanced therapy medicinal products, and their production thus requires the use of medicinal manufacturing procedures that do not or minimally alter the biological features of the cells. In this study, we designed an original *ex vivo* protocol for the isolation, long-term cryopreservation, and short-term amplification of HDPCs that includes enzyme-free cell selection and the use of xenogeneic-free products and SFM. This protocol is easy, safe, fast, and cost-effective, and it allowed for the production of a clinical scale number of mesenchymal pulp cells maintaining their osteo/odontoblastic differentiation potential.

As was previously shown for the isolation of human adipose or Wharton jelly stem/progenitor cells, minimal manipulation leads to higher safety and efficacy of cell production (15, 16). In this study, we selected exclusively impacted third molars between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) to minimize the risk of pulp tissue contamination with oral microorganisms and to avoid the cell stress that results from crown root mechanical separation during the pulp recovery from older teeth (4, 14). HDPC isolation at around the crown-completed stage

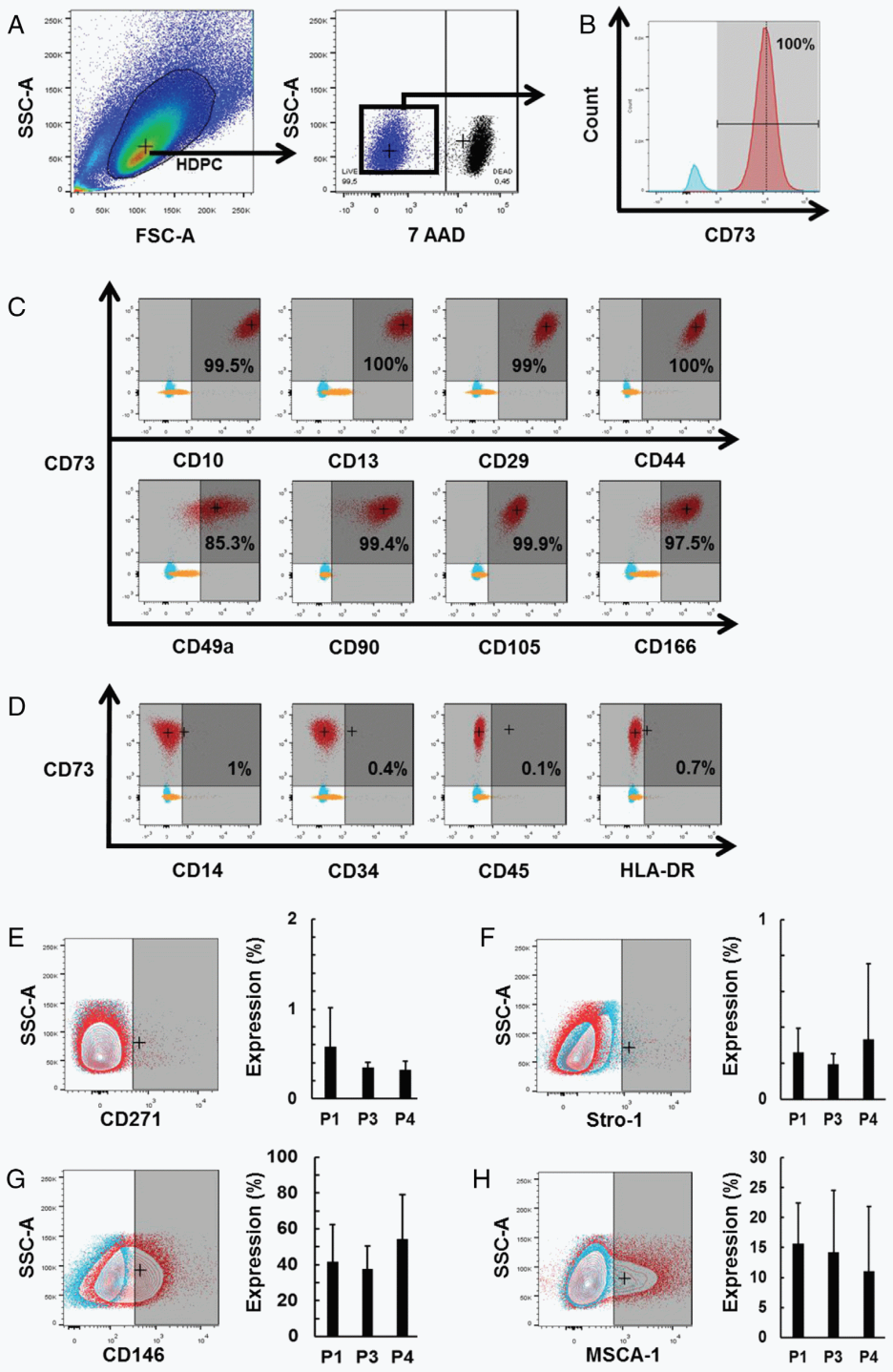

Figure 2. Immunophenotypic analysis of P1-HDPC cultured in SFM-1 with multicolor flow cytometry. The gating tree was set as follows: (*A, left*) Initial gating (black polygon) representing the cell distribution based on size (FSC-A) and intracellular composition (SSC-A); (*A, right*) The gate was then set on 7AAD-negative live cells, colored in blue. (*B*) All HDPC expressed the cell surface marker CD73 (*red peak*) which was therefore chosen as a basis versus additional markers. *Blue peak*: isotype control. (*C*) Almost all CD73-positive HDPC (≥97%) expressed the mesenchymal markers CD10, CD13, CD29, CD44, CD90, CD105 and CD166, whereas CD49a was expressed by a lower number of cells (85.3%). (*D*) The hematopoietic markers CD14, CD34, CD45 and HLA-DR were expressed by very few cells (≤1%). Data from (*A*), (*B*), (*C*), and (*D*) are representative of five independent experiments. Expression levels of the stem/progenitor cell markers CD271 (LNGFR) (*E*), Stro-1 (*F*), CD146 (*G*), and MSCA-1 (*H*) were maintained during cell passaging and did not differ significantly ($n = 5$). Error bars: mean ± SD.

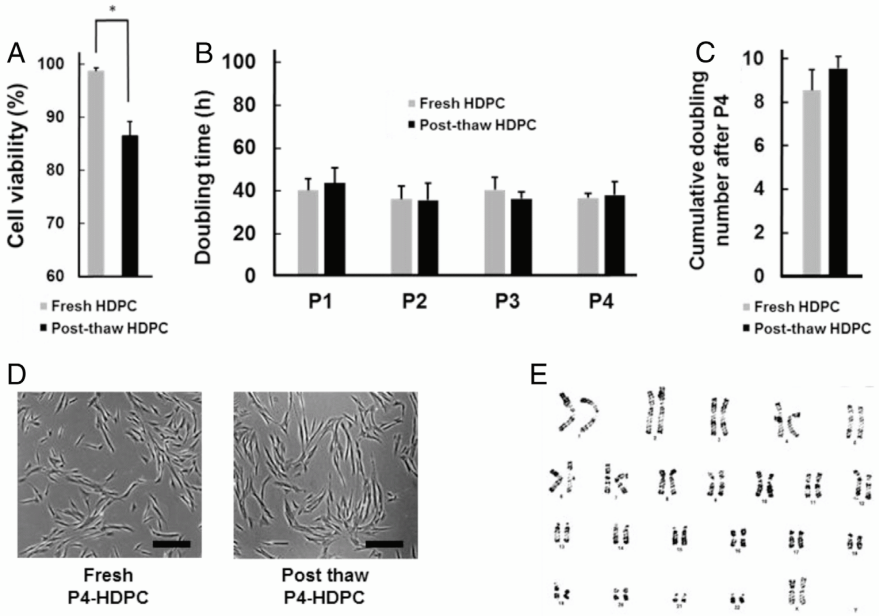

Figure 3. Post-thaw HDPC amplification in SFM-1. (*A*) The viability of post-thaw HDPCs was significantly decreased compared with freshly isolated cells ($n = 5$). (*B*) Cell DTs were stable and similar from P1 to P4 (≈ 40 hours) for both populations ($n = 5$). (*C*) CDNs at P4 were similar (≈ 9) ($n = 5$). (*D*) The morphology of post-thaw was fibroblastlike and similar to that of fresh HDPCs. (*E*) Post-thaw P4-HDPCs showed a normal karyotype on G-band analysis (the karyotype shown is representative from 4 patients). $*P < .05$. Error bars: mean $\pm$ standard deviation. Scale bar = 100 μm.

was previously associated with short cell DTs and high growth rate (17), and we obtained cell DTs and growth rates similar to those reported by these authors.

Two main methods have been described for isolating cells from tissues: explant culture and mechanical/enzymatic disruption associated with either cell adhesion on a plastic dish or cell sorting (3, 18). However, because of the longer handling time compared with explant cutting and the possible consequences of the tissue enzymatic dissociation on cell fate, enzymatic dissociation of tissues appears not to be adapted to medicinal manufacturing (19). In addition, tissues and cells exposed to collagenase, an enzyme frequently used to recover HDPCs from the pulp tissue, are considered to be "more than minimally manipulated" by the Food and Drug Administration. Likewise, cell selection by sorting procedures appears to not be ideal because of the prohibitive cost and complexity of the technique (7, 20). On the contrary, the minimal, easy, and stressless cell handling brought by the explant culture appears to be a beneficial procedure for isolating HDPCs (21).

Cell production under good manufacturing practices procedures implies the use of xeno-free materials and reagents to prevent the risk of viral, bacterial, or prion contamination and the possible induction of immunizing effects in the final recipient (11, 22). For this reason, xeno-free dissociating reagents such as TrypLe or Accutase (Life Technologies, Carlsbad, CA) have been recommended for cell passaging instead of the xenogeneic animal trypsin commonly used (23, 24). Likewise, if cell culture medium supplementation with xeno- or allogeneic serum remains permitted, it should be limited to "cases for which a valid alternative cannot be found" (European directive 1394/2007). Indeed, in addition to the risk of pathogen contamination and induction of immunizing effects, industrial production of serum is associated to product variability, and the serum itself can promote early cell differentiation (11, 22). For these reasons, we decided to use SFM. HDPC culture in SFM was previously reported, but results are not directly comparable because cells were isolated by enzymatic dissociation of the pulp tissue and by using a serum-containing medium (25). HDPC isolation in SFM was previously associated with a lack of proliferation, and, to the best of our knowledge, we are the first to isolate, cryopreserve, and efficiently amplify HDPCs entirely in serum-free and xenogeneic-free conditions (26).

Several authors have shown that *ex vivo* cultured cells can include several populations such as stem/progenitor cells, fibroblasts, and perivascular cells (1, 18, 21, 27). Our multiparametric immunophenotypic analysis of specific surface markers indicated that more than 97% isolated HDPCs presented a mesenchymal profile. We also observed variable expression of cell surface molecules that were previously described as mesenchymal stem cell/progenitor markers (20, 28). Indeed, we detected the expression of CD146 and MSCA-1 by about 40% and 15% of HDPCs, respectively, whereas CD271 and Stro-1 were expressed by a very low number of cells. These results suggest the presence of distinct cell populations in the cultured HDPCs. The proportion of cells expressing Stro-1 and CD271 was low and similar to that reported by others in explant cultures (21). The perivascular marker CD146 has been related to high clonogenicity and multipotency, and the large number of cells expressing CD146 in our cultures might reveal/characterize an HDPC cell state (28). We report an average expression of MSCA-1 by cultured HDPCs similar from P1 to P4. Because this marker is related to high mineralized tissue cell differentiation potential in human bone marrow stem/progenitor cells, it may be used for selecting and monitoring HDPC populations having this capacity (29, 30). Studies are ongoing in our laboratory to test this possibility.

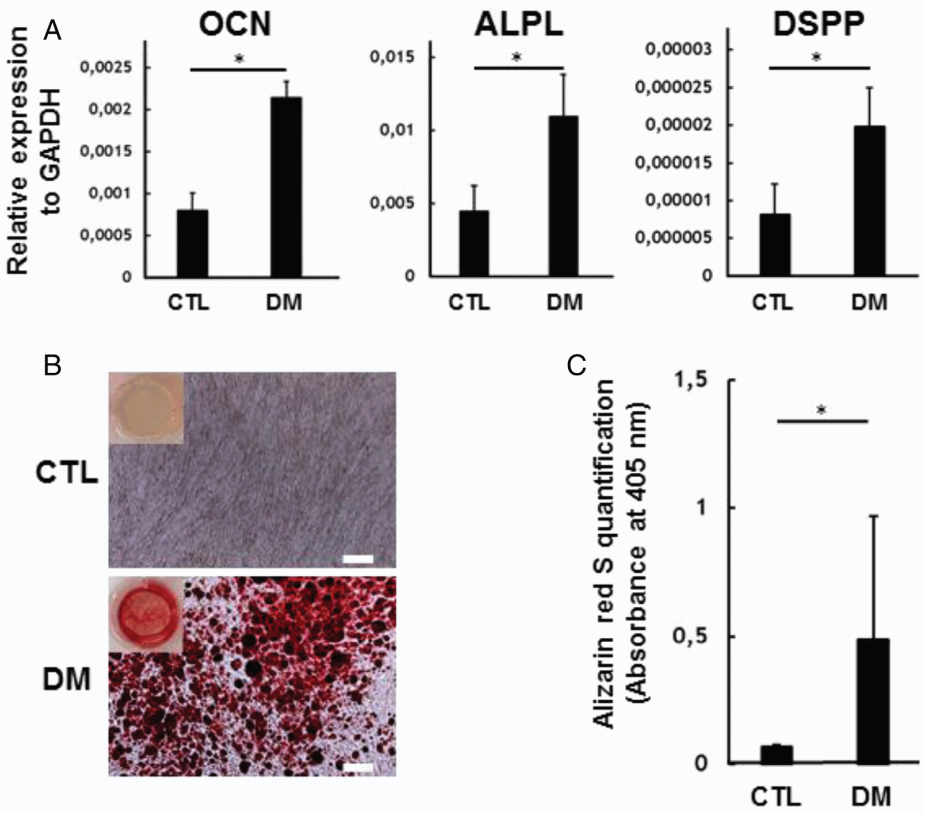

Figure 4. Determination of the osteo/odontoblast differentiation potential of post-thaw HDPCs. (*A*) The expression of osteocalcin (OCN), alkaline phosphatase (ALPL), and dentin sialophosphoprotein (DSPP) genes was significantly increased after 4 weeks of culture in the differentiation medium compared with cells maintained in the amplification (control) medium ($n = 4$). (*B*) ARS staining and (*C*) quantification showed a higher mineralization degree in HDPC cultures maintained in the differentiation medium compared with cultures performed in the control medium ($n = 4$). *$P < .05$. Error bars: mean ± standard deviation. Scale bar = 100 μm. CTL, control medium; DM, differentiation medium.

Prolonged *ex vivo* amplification of human cells is currently not recommended to limit the risk of modifying the phenotype of HDPCs isolated from the explants and preventing their future differentiation into cells capable of forming mineralized tissues (10, 18, 31). In this context, culture conditions that maintain the cell fate with the possibility to rapidly obtain clinical scale numbers of cells are required (9). Our HDPC cultures in SFM-1 gave rise to about 10^6 cells from 1 pulp after 2 weeks of explant culture (not shown). This result is comparable with that obtained for dental pulp stem cells in serum-containing medium (5). Importantly, because the cell DTs remained constant from P1 to P4 (≈40 hours), we calculated that more than 25.10^7 cells could be theoretically obtained after 4 passages with 1 pulp, which is likely to be a sufficient number for 1 pulp regeneration, 1 bone socket filling after tooth extraction, or 1 localized periodontal treatment (13, 32). Interestingly, the average number of cells expressing each of the 4 mesenchymal stem cell/progenitor markers remained similar from P1 to P4, suggesting that the cell fate was not profoundly affected by our culture conditions. Our results further indicate that cryopreservation in SFM had no negative impact on cell DTs and cumulative cell numbers. Although the viability of cells cryopreserved in SFM was decreased compared with fresh cells, it remains similar to that previously reported (33). Importantly, rapid amplification of post-thaw HDPCs up to P4 did not lead to genomic instability, which would be potentially harmful for the recipient patient.

Cell culture on rigid plastic dishes can potentially alter cell fate, and the latter is better maintained on extracellular matrix–coated surfaces (12, 34). Accordingly, we precoated the culture dish surface with human placental collagens I and III that were chosen because they are the 2 most abundant collagens in the dental pulp extracellular matrix. Moreover, it was previously reported that HDPC culture was successfully performed on collagen surfaces (35), so we postulated that collagen offered an appropriate surface to maintain the fate of cells outgrowing from the pulp explants and their differentiation potential. Osteo/odontoblast stem/progenitor cells isolated from human dental pulp were recently successfully used for pulp/dentin and bone engineering (6, 7). Interestingly, we found that HDPCs cultured in a specific differentiation medium after short-term amplification clearly expressed an osteo/odontoblast differentiation potential.

In summary, we designed, in this study, specific conditions for HDPC isolation, storage, and amplification with a medicinal manufacturing approach. These conditions include minimal tissue manipulation, enzyme-free isolation, use of xenogeneic-free products, and SFM culture. They allow for the expression of stem/progenitor cell markers and preserve amplification kinetics without inducing karyotype abnormality while maintaining the differentiation potential of HDPCs into osteo/odontoblast cells. These findings may constitute a basis on which to investigate how HDPC production can be optimized for human pulp/dentin and bone tissue engineering.

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The authors deny any conflicts of interest related to this study.

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Discussion et conclusion de l'article 3 :

Aujourd'hui, les produits cellulaires destinés à l'ingénierie de la pulpe dentaire humaine sont référencés comme « Produits médicamenteux de thérapie avancée », avec des recommandations concernant des protocoles de culture cellulaire pour assurer une reproductibilité, une efficacité et une sécurité optimale des produits destinés aux patients. Dans ce contexte, la très grande majorité des protocoles rapportés dans la littérature ne sont pas satisfaisants en ce qui concerne la production des CPD (Tirino & Papaccio 2012)(La Noce et al. 2014)(Nakashima & Iohara 2014)(Mayo et al. 2014)(Albuquerque et al. 2014)**.** Afin de pallier ces inconvénients, nous avons développé un nouveau protocole de production des CPD avec une approche de BPF : sélection du stade de développement des troisièmes molaires (stades 5 à 7 de la classification de Nolla), isolement non-enzymatique des cellules à partir du tissu pulpaire (culture d'explants), limitation à 4 du nombre de passages, culture et cryoconservation en milieu sans sérum, absence d'utilisation de produits xénogéniques (trypsine porcine par exemple). Les cultures sont réalisées sur un substrat recouvert, à parts égales, de collagènes de types I et III humains qui sont les collagènes majoritaires de la matrice extracellulaire pulpaire et qui constituent le substrat naturel des cellules mésenchymateuses pulpaires *in vivo*. L'importance de recréer *in vitro* pour les CPD un microenvironnement proche de celui *in vivo* a encore été rappelée récemment, car il permet de maintenir le phénotype des cellules (Smith et al. 2015).

Notre protocole a permis d'isoler, d'amplifier rapidement une population de cellules à partir de culture d'explants de pulpe dentaire humaine. L'immunophénotypage de ces cellules a montré un profil mésenchymateux (expression des marqueurs moléculaires CD10, CD13, CD29, CD44, CD49a, CD73, CD90, CD105 et CD166). Un fort pourcentage de cellules exprime également les marqueurs de CSM CD146 (40% des cellules) et MSCA-1 (15%). Ce profil reste stable au moins jusqu'au 4ème passage, passage au-delà duquel il n'est plus recommandé d'utiliser des cellules amplifiées *ex vivo* (Torre et al. 2015).

Nous avons pu cryopréserver ces cellules pendant au moins 510 jours. Nous avons observé que leur viabilité après décongélation est légèrement inférieure à celle des cellules fraichement isolées. Toutefois, les temps de doublement des deux populations sont stables et similaires (# 40 heures), ce qui permet dans les deux cas d'obtenir après amplification des quantités de cellules importantes. Les cellules amplifiées ne montrent pas d'anomalie caryotypique après 4

passages. Elles sont capables de se différencier en cellules ostéo/odontoblastiques lorsqu'elles sont cultivées pendant 4 semaines dans un milieu inducteur spécifique.

Sur le plan quantitatif, notre protocole permet d'obtenir plusieurs dizaines de millions de cellules à partir d'une seule pulpe dentaire, ce qui est compatible avec la régénération d'un complexe dentinopulpaire dans l'endodonte d'une dent humaine ou avec le comblement d'un défaut osseux de petite étendue, voire de taille moyenne, comme par exemple d'une poche parodontale ou d'une alvéole dentaire (Yamada et al. 2013).

L'ensemble de ces résultats indique que le protocole que nous avons développé permet de sélectionner, d'amplifier rapidement et de manière importante, avec une approche de BPF, une population qui possède des propriétés ostéo/odontoblastiques. Pour confirmer ces résultats, une approche de culture tridimensionnelle, qui se poursuivra par une étude *in vivo* chez l'animal, est actuellement en cours d'investigation dans notre laboratoire (voir la partie Perspectives).

Article 4 : Description d'une procédure standardisée pour le prélèvement, l'isolement et la caractérisation de cellules souches/stromales mésenchymateuses de la pulpe dentaire et de la gelée de Wharton avec un minimum de manipulation

A standardized procedure to obtain mesenchymal stem/stromal cells from minimally manipulated dental pulp and Wharton's jelly samples.

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Résumé : Un défi majeur de la régénération tissulaire est l'isolement de cellules à partir d'une source peu ou pas invasive pour le patient et selon les recommandations de bonnes pratiques de fabrication. Les tissus et les cellules doivent ainsi être le moins « manipulés » possible et cultivés en présence de milieux contrôlés. Dans ce contexte, les troisièmes molaires en phase pré-éruptive et la gelée de Wharton présentent un grand intérêt car elles sont prélevées selon des protocoles peu traumatisants et car elles contiennent des cellules qui peuvent être facilement amplifiées *ex vivo*, permettant d'obtenir une quantité de cellules suffisante pour être utilisable cliniquement. L'objectif de ce travail est de mettre au point des procédures standardisées pour la collecte de la pulpe dentaire et de la gelée de Wharton, et pour l'isolement et la caractérisation de cellules souches/stromales mésenchymateuses (CSMs) qu'elles contiennent. La pulpe dentaire a été collectée à partir de dents de sagesse (stades 5 à 7 de Nolla) et la gelée de Wharton a été isolée à partir de cordons ombilicaux. Des CSM-PD et des CSM-GW ont été obtenues respectivement à partir d'explants de Pulpe dentaire et de Gelée de Wharton cultivés pendant deux semaines. Les CSMs ont alors été analysées par immunophénotypage grâce à un cytomètre de flux. Ce protocole standardisé pour la pulpe dentaire et la gelée de Wharton nous a permis d'isoler des cellules pour les deux tissus. L'immunophénotypage des CSMs en culture révèle un profil mésenchymateux quasi identique pour le panel de récepteurs que nous avons utilisé. Ces résultats suggèrent que la mise en culture d'explants tissulaires de pulpe dentaire et de gelée de Wharton selon le même protocole permet d'obtenir des populations de cellules mésenchymateuses proches. Des investigations avec un nombre plus important de marqueurs de surface permettront de déterminer si ces populations sont identiques ou possèdent des caractéristiques différentes, et si elles contiennent des sous-populations cellulaires.

TECHNICAL NOTE

A STANDARDIZED PROCEDURE TO OBTAIN MESENCHYMAL STEM/STROMAL CELLS FROM MINIMALLY MANIPULATED DENTAL PULP AND WHARTON'S JELLY SAMPLES

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Keywords

Human dental pulp, Wharton's jelly, stem/stromal cells, good manufacturing practices, cell-based medicinal products, immunophenotyping

Introduction

Transplantation of mesenchymal stem/stromal cells (MSCs) has emerged as an effective method to treat diseased or damaged organs and tissues, and hundreds of clinical trials using MSCs are currently under way to demonstrate the validity of such a therapeutic approach. However, most MSCs used for clinical trials are prepared in research laboratories with insufficient manufacturing quality control. In particular, laboratories lack standardized procedures for *in vitro* isolation of MSCs from tissue samples, resulting in heterogeneous populations of cells and variable experimental and clinical results.

MSCs are now referred to as Human Cellular Tissue-based Products or Advanced Therapy Medicinal Products, and guidelines from the American Code of Federal Regulation of the Food and Drug Administration (21 CFR Part 1271) and from the European Medicines Agency (European Directive 1394/2007) define requirements for appropriate production of these cells. These guidelines, commonly called "Good Manufacturing Practices" (GMP), include recommendations

about laboratory cell culture procedures to ensure optimal reproducibility, efficacy and safety of the final medicinal product. In particular, the Food and Drug Administration divides *ex vivo* cultured cells into “minimally” and “more than minimally” manipulated samples, in function of the use or not of procedures “that might alter the biological features of the cells”. Today, minimal manipulation conditions have not been defined for the collection and isolation of MSCs (Torre et al. 2015)(Ducret et al. 2015). Most if not all culture protocols that have been reported so far are unsatisfactory, because of the use of xeno- or allogeneic cell culture media, enzymatic treatment and long-term cell amplification that are known to alter the quality of MSCs.

The aim of this study was to describe a standardized procedure for recovering MSCs with minimal handling from two promising sources, the dental pulp (DP) and the Wharton’s jelly (WJ) of the umbilical cord. The quality and homogeneity of the expanded cell populations were assessed by using flow cytometry with criteria that go beyond the International Society of Cellular Therapy (ISCT) guidelines for MSC characterization.

Materials and Methods

Tissue collection

Healthy, impacted human third molars were collected from donors aged 13-17 years **(Fig 1.A)**. Only teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were used **(Fig 1.B)**.

Umbilical cords were collected either before or after the placenta was delivered **(Fig 1.F)**. A section of the umbilical cord between 10 and 25 cm long was cut **(Fig 1.G)**.

Tissues were placed in a 50 mL tube supplemented with 1% antibiotics (PSA = Penicillin-Streptomycin-Amphotericin B, Lonza) and transported to the laboratory within 12 hours.

Cell isolation

Dental pulps were aseptically, gently extirpated from pulp chambers with fine tweezers. The apical part of the radicular pulp was removed with a scalpel to prevent contamination by dental papilla and periapical cells **(Fig 1.C)**. Pulps were then rinsed with PBS, placed onto a sterile glass slide and cut with a scalpel into 0.5 to 2 mm^3 fragments to form explants **(Fig 1.D)**.

The umbilical cord fragment was shaken thoroughly to remove the remaining blood and potential microbial contaminants and then transversally cut into 1 to 2 mm-thick slices **(Fig 1.H). C**ircular blades were then used to isolate 3 to 4 standardized WJ pieces of 2.5 mm diameter and 1 to 2 mm thickness **(Fig 1.I)**.

Four explants of each of the tissues were seeded per well on 6-well plates pre-coated with an equal mixture of human placental collagens I and III at a final concentration of 0.5 µg/cm^2 (ABCellBio, Paris, France) and then cultured in serum-free SPE-IV defined medium (ABCellBio®, Paris, France) supplemented with antibiotics. The culture medium was changed twice a week. At confluence, cells were detached with a xeno-free recombinant protease (TrypLE Select 1X, Life Technologies), seeded at 5 x 10^3 cells/cm^2 and cultured for one week to obtain a sufficient number of cells for immunophenotyping.

Immunophenotypic characterization

Cultured cells were immunophenotyped after one passage as previously described (Ducret, Fabre, Farges, et al. 2015). Briefly, 3.10^6 cells were stained with 17 fluorochrome-conjugated antibodies (Table 1). The nucleic acid dye 7AAD (7-Amino-Actinomycin D, BD-Biosciences, Le Pont de Claix, France) was used for the exclusion of non-viable cells. Samples were acquired on a BD FACSCanto II Flow cytometer (BD Biosciences) as uncompensated events and then recorded as FCS 3.0 files. Analysis and compensation were performed using the FlowJo vX software. The percentage of cells positively stained corresponded to the percentage of cells present within a gate established so that less than 1% of the measured positive events represented nonspecific binding by the fluorochrome-conjugated isotype-matched control.

Results

Cells started to grow from the cultured DP explants after 3-4 days (Figure 1.E). Two weeks later, pooling of outgrowing cells from one dental pulp allowed the harvesting of about 10^6 cells. Cells started to migrate from the WJ explants after 4-5 days (Figure 1.J). The confluence was reached after about two weeks for DP cultures and three weeks for WJ cultures. All DP and WJ cells analyzed failed to express exclusion markers for hematopoietic cells (CD45), endothelial cells (CD31), monocytes/macrophages (CD14), B cells (CD79α), hematopoietic progenitors (CD34) and activated immune cells (HLA-DR). On the contrary, WJ and DP isolated cells were positive to the MSC markers recommended by ISCT (CD73, CD90, CD105 and HLA-ABC), as well as other, now recognized MSC markers (CD10, CD13, CD29, CD44, CD49a and CD166). DP and WJ cell populations showed similar profiles (Figure 2). Results shown are representative from 10 independent samples, which indicates the reproducibility of our procedure.

Discussion

Since the discovery of DP- and WJ-MSCs more than one decade ago (Gronthos et al. 2000)(Wang et al. 2004), numerous papers have reported the isolation of mesenchymal stem/stromal cells from DP and WJ. However, guidelines for standardized procedures are lacking. For exemple, there are no rules specifying the tooth development stage for pulp MSC collection. Transport from the operating block to the laboratory requires a sterile medium and it was previously shown that DP-MSCs remain viable in phosphate-buffered saline (PBS) (Woods et al. 2009). We show here that the same procedures can be successfully applied to the transport of umbilical cord samples. Additionally, samples were processed within 12 hours of collection to prevent hypoxic stress and microbial contaminations.

Two main techniques have been described for the isolation of MSCs (Hilkens et al. 2013): The direct culture of tissue fragments (explants), which has been used in this study, and the dissociation of the tissue by proteolytic enzymes that digest the extracellular matrix meshwork and free resident cells that can be immediately plated and cultured. The explant method was repeatedly proven to be similar to enzymatic digestion (Hilkens et al. 2013). Explant culture has the advantages of avoiding the use of proteolytic enzymes that are suspected to alter surface cell receptors and of providing a more homogeneous morphology of recovered cells (Ducret et al. 2015). The diminution of the number of enzymatic components getting in contact with the cells is also more compliant with cGMP approaches.

The identity of cultured cells is generally determined by flow cytometry analysis of surface antigens. During the past decade, MSCs have been mostly identified according to the recommendations of the International Society of Cellular Therapy (ISCT) (Dominici et al. 2006). However, it is today admitted that several markers initially considered by ISCT as being characteristic of MSCs (for instance CD73, CD90 and CD105) are shared by several other populations including progenitor cells, mature fibroblasts and/or perivascular cells (Alt et al. 2011)(Halfon et al. 2011)(Lv et al. 2014). Here, in spite of the use of additional MSC markers, we failed to evidence differences between the cell populations from DP and WJ in 10 independent samples. This suggests that DP and WJ cells isolated and cultured according to the same procedure might lead to the recovery of similar mesenchymal cell populations. Additional studies are ongoing in our laboratory to improve the characterization and compare the immunophenotypic profile of mesenchymal cells isolated from various human tissues according to the procedure described here.

Conclusions and perspectives

We described in this study standardized procedures by using minimally manipulated tissue samples for the collection and isolation of MSCs recovered from DP and WJ. This approach relies on commercially available serum-free medium, culture dish coating with a collagen solution and medical grade xenogeneic enzymes. Further investigations with a greater number of membrane markers are required to determine if DP and WJ populations are identical or possess different features and if subpopulations are present in both these populations. Indeed, in a recent immunophenotypic analysis, we found that mesenchymal dental pulp cells isolated similarly to this study contained cells a percentage of whom expressed the stem cell/progenitor markers CD146 and MSCA-1 (Ducret et al. 2015).

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Lv, F.J. et al. (2014). “Concise Review: The surface markers and identity of human mesenchymal stem cells.” *Stem Cells* 32(6):1408–19.

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Wang, H.S. et al. (2004). “Mesenchymal Stem Cells in the Wharton’s Jelly of the Human Umbilical Cord.” *Stem Cells* 22(7):1330–37.

Woods, E.J. et al. (2009). “Optimized Cryopreservation Method for Human Dental Pulp-Derived Stem Cells and Their Tissues of Origin for Banking and Clinical Use.” *Cryobiology* 59(2):150–57.

Target	Format	Vendor	Isotype	Reference
NA (viability)	7AAD	BD Biosciences	NA	559925
CD10	PE	BD Biosciences	IgG2a, κ	555375
CD105	PE	BD Biosciences	IgG1, κ	555487
CD13	APC	BD Biosciences	IgG1, κ	557454
CD14	APC-H7	BD Biosciences	IgG2b, κ	560180
CD166	PE	BD Biosciences	IgG1, κ	559263
CD29	APC	BD Biosciences	IgG1, κ	559883
CD31	FITC	BD Biosciences	IgG1, κ	555445
CD34	APC	BD Biosciences	IgG1, κ	555824
CD44	APC-H7	BD Biosciences	IgG2b, κ	560532
CD45	V500	BD Biosciences	IgG1, κ	560777
CD49a	PE	BD Biosciences	IgG1, κ	559596
CD73	PE-Cy7	BD Biosciences	IgG1, κ	561258
CD79a	BV421	BD Biosciences	IgG1, κ	562852
CD90	FITC	BD Biosciences	IgG1, κ	555595
D7-Fib	PE	Antibodies-online	IgG2a, κ	ABIN319868
HLA ABC	V450	BD Biosciences	IgG1, κ	561346
HLA DR	V500	BD Biosciences	IgG1, κ	561224

Table 1: Antibodies used for the immunophenotypic analysis.

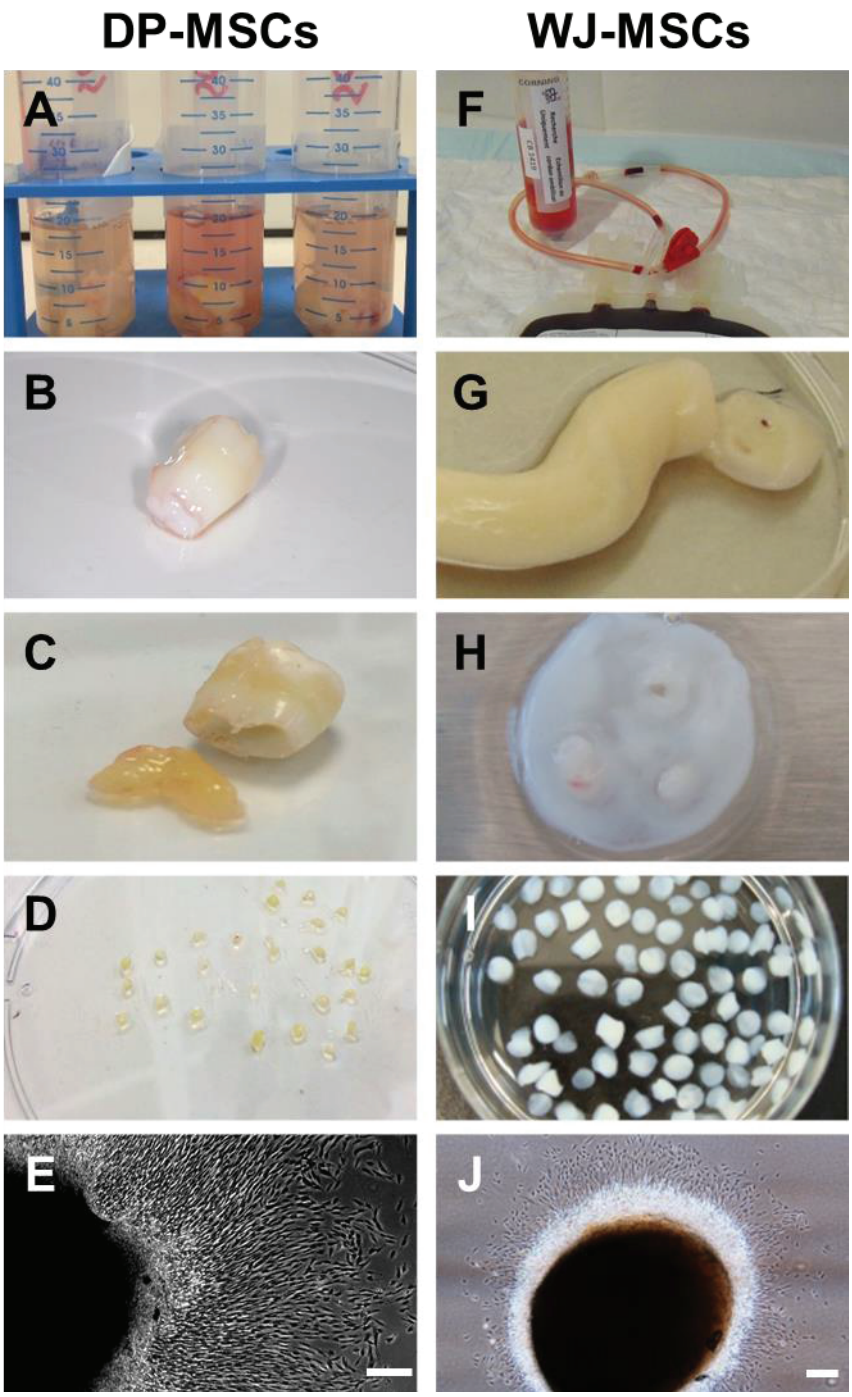

Figure 1: Standardization of the isolation process for DP- and WJ-MSCs.

DP (A,B) and UC (F,G) were processed for isolation by gently extirpating the pulp from the tooth (C), or by slicing the UC (H), and further cutting (D) or punching (I) of the mesenchymal tissue, respectively, to obtain explants. E and J show cells outgrowing from the DP and WJ explants, respectively, 96 h after seeding. Scale bar: 200 µm

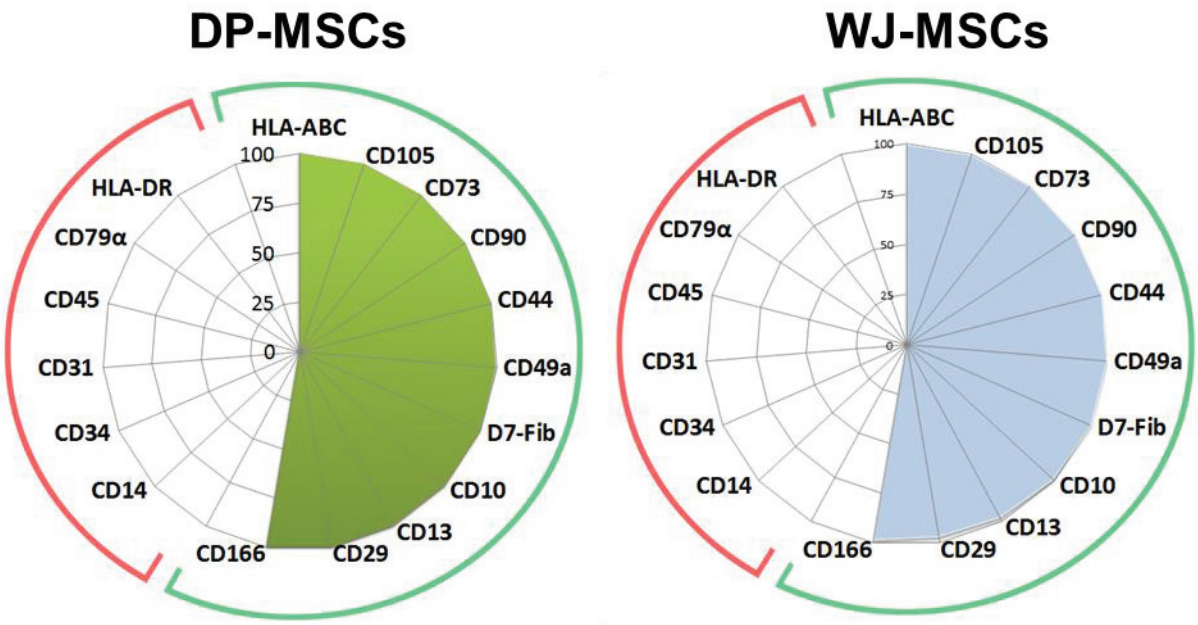

Figure 2: Identity cards of DP- and WJ-MSCs.

Sonar representation of flow cytometry results showing the frequencies of positive (green arc) and negative (red arc) markers for DP- and WJ-MSCs, respectively. Cell immunophenotype after one passage. Data shown are representative of 10 DP and 10 WJ samples.

Synthèse et conclusion de l'article 4 :

Un défi majeur de la régénération du complexe dentinopulpaire est l'isolement de CSM à partir d'une source peu ou pas invasive pour le patient. Dans ce contexte, les troisièmes molaires en phase pré-éruptive présentent un grand intérêt car elles sont extraites par les chirurgiens-dentistes à la fin des traitements orthodontiques selon des protocoles peu traumatisants. Elles contiennent diverses populations de CSM pulpaires qui peuvent être efficacement amplifiées *ex vivo* afin d'obtenir une quantité de cellules suffisantes pour être utilisables en clinique. Cependant, comme nous l'avons souligné dans l'article n°2 et comme le rappelle une récente revue sur l'isolement des CSM, il n'existe pas ou peu de protocole standardisé pour la collecte et l'isolement de cellules souches/stromales mésenchymateuses à partir de la pulpe dentaire humaine (Ducret et al. 2015)(Torre et al. 2015).

Ainsi l'objectif de notre travail était de mettre au point une procédure standardisée pour la collecte des dents et l'isolement de cellules à partir de la pulpe dentaire (Figure 27). Pour valider la qualité de notre protocole, nous avons comparé les CSM-PD avec des CSM isolées d'un autre tissu prélevé de manière non invasive, la gelée de Wharton (CSM-GW)(Mueller et al. 2014).

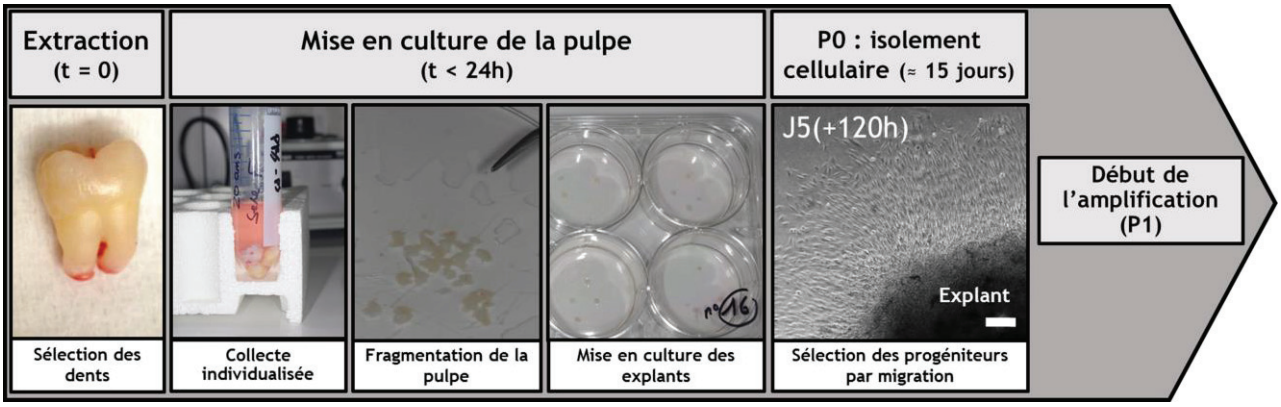

Figure 27 : Procédure pour l'isolement CSM-PD. Les dents sont sélectionnées après l'extraction lors que le stade de développement de 5 à 7 est confirmé. Elles sont ensuite collectées et transportées jusqu'au laboratoire. Après séparation de la pulpe et de la papille apicale, la pulpe est fragmentée en 20 à 25 fragments qui sont placés sur des boites de culture recouvertes d'un mélange de collagène I et III. Apres 3 à 5 jours, les premières CPD migrent de l'explant et après deux semaines, la phase d'amplification peut débuter avec la réalisation d'un premier passage.

Nos résultats indiquent que le protocole que nous avons développé permet, de manière similaire aux CSM-GW, de collecter des dents de manière stérile et d'isoler rapidement et de manière

importante des CSM-PD. Ces cellules présentent un profil membranaire de type mésenchymateux similaire à celui des CSM-GW. Une investigation plus large est en cours de réalisation dans notre laboratoire avec un nombre plus important de récepteurs et différentes sources de cellules souches/progénitrices (Figure 28).

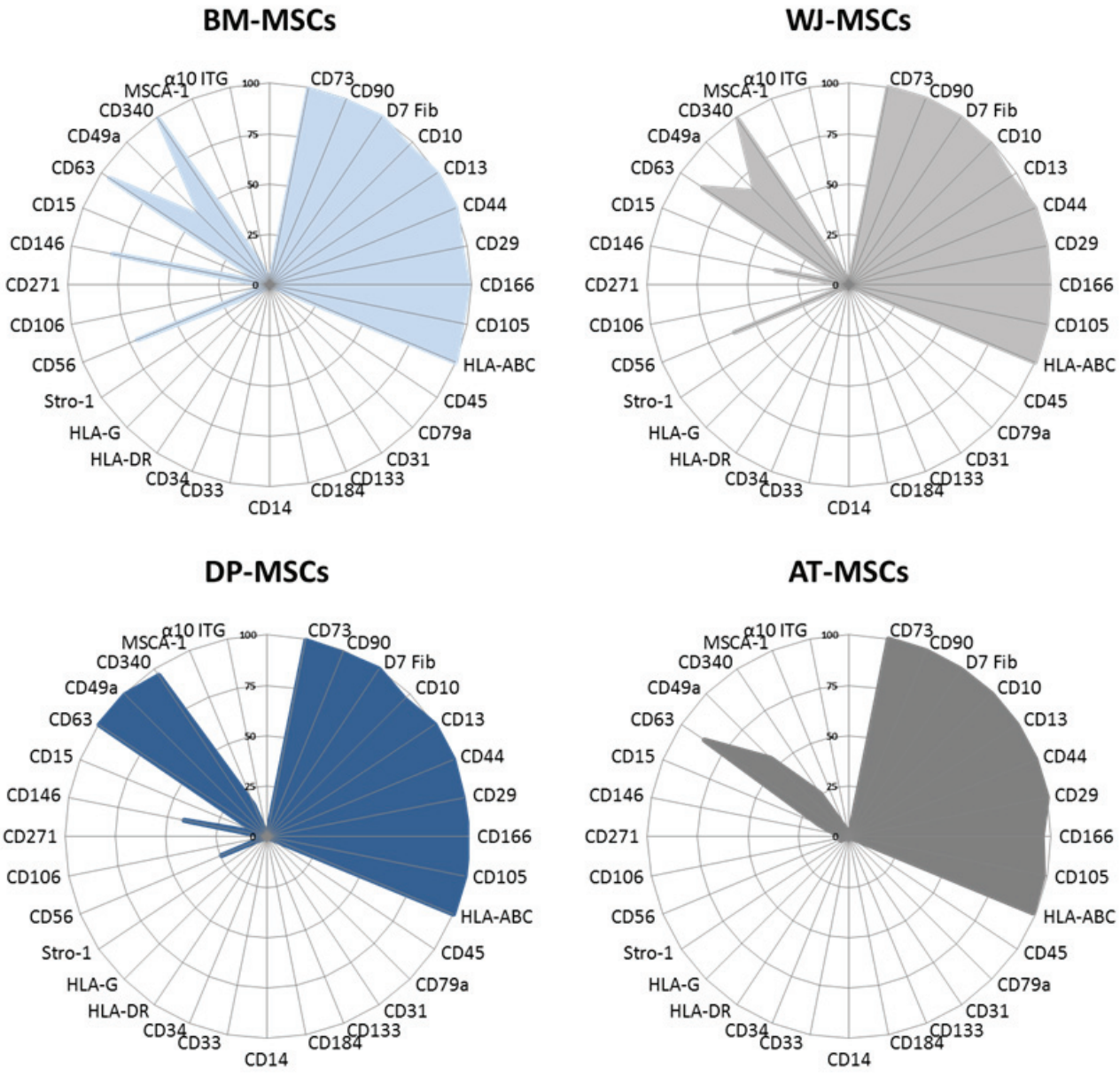

Figure 28 : Immunophénotypage membranaire des CSM(MSCs) issues de la moelle osseuse (BM), de la gelée de wharton (WJ), de la pulpe dentaire (PD) et du tissu adipeux (AT). Représentation en sonar de cellules isolées et amplifiées pendant un passage dans le milieu SPE-IV (ABCellBio). Fabre, Ducret, et al. Article en préparation.

CONCLUSION GENERALE

L'objectif de ce travail était de déterminer des conditions favorables au développement futur d'un MTI permettant de régénérer un complexe dentinopulpaire dans l'endodonte après exérèse du tissu pulpaire lésé de manière irréversible, désinfecté et mis en forme. En effet, le traitement actuel, qui consiste à remplacer le tissu pulpaire par un biomatériau à base de gutta-percha présente des limites majeures parmi lesquelles le manque d'étanchéité du biomatériau vis-à-vis des bactéries présentes dans la cavité buccale.

Plusieurs approches innovantes de médecine régénérative à base de CSM adultes ont récemment fait leurs preuves dans la réparation et le remplacement des tissus endommagés chez l'homme (Demoor et al. 2014). De telles approches présentent un grand intérêt en médecine bucco-dentaire car la régénération d'un tissu pulpaire fonctionnel dans l'endodonte permettrait à la dent de se défendre face à des agressions futures (Cao et al. 2015). Elle favoriserait également la formation d'une barrière de dentine tertiaire au contact du matériau bioactif dentino-inducteur. Cette barrière représente la meilleure stratégie d'éloignement et de protection de la pulpe vivante saine vis-à-vis du milieu buccal septique.

Pour relever ce défi, il est nécessaire de collecter et d'amplifier des cellules, de manière à produire un médicament de thérapie innovante sur mesure.

Pour développer un produit répondant aux exigences d'une application clinique, nous avons donc :

- mis au point, un protocole innovant qui cherche à respecter le plus possible les BPF actuelles.
- isolé de manière non-enzymatique, amplifié et congelé des cellules mésenchymateuses pulpaires en utilisant des milieux sans sérum et en l'absence de produits xénogéniques.
- effectué un travail de caractérisation de ces cellules afin de confirmer la présence de cellules souches/progénitrices.
- confirmé le potentiel de différenciation ostéo/odontoblastique de ces cellules dans nos conditions de culture.

Les perspectives de notre travail sont les suivantes :

- Poursuivre la caractérisation des sous-populations de CPD identifiées *in vitro* dans l'article 2 et qui expriment les marqueurs de cellules souches/stromales mésenchymateuses MSCA-1 et/ou CD146. Etudier la cinétique d'expression de ces marqueurs membranaires. Trier les sous-populations cellulaires identifiées et étudier leur comportement *in vitro* dans un environnement tridimensionnel. Analyser les effets d'un enrichissement après tri cellulaire qui pourrait permettre de sélectionner certains progéniteurs pour améliorer la qualité du tissu formé.

- Développer un modèle *in vivo* chez le petit animal, dans un environnement tridimensionnel qui permette aux cellules de survivre, d'adhérer, de proliférer et de se différencier en cellules pulpaires (fibroblastes, odontoblastes,…).

PERSPECTIVES

- **Immunophénotypage et tri cellulaire de progéniteurs issus de la pulpe dentaire humaine pour la régénération des tissus squelettiques**

Projet lauréat de l'Institut Français de Recherche Odontologique (IFRO) en 2014

L'immunophénotypage membranaire des sous-populations de cellules pulpaires présentes dans notre modèle de culture a permis d'établir une carte d'identité à P1 de ces cellules et d'analyser la cinétique d'expression de marqueurs membranaires plus spécifiques (CD146, MSCA-1 [ou TNAP], CD271 et Stro-1) à différents temps de culture (P0, P1, P3…)(Figure 29). Cet immunophénotypage, associé à l'analyse multiparamétrique des données, indique que certains marqueurs ou certaines combinaisons de marqueurs pourraient permettre de caractériser des sous-populations de cellules pulpaires possédant des propriétés accrues de différenciation ostéo/odontoblastique et de formation de tissu minéralisé.

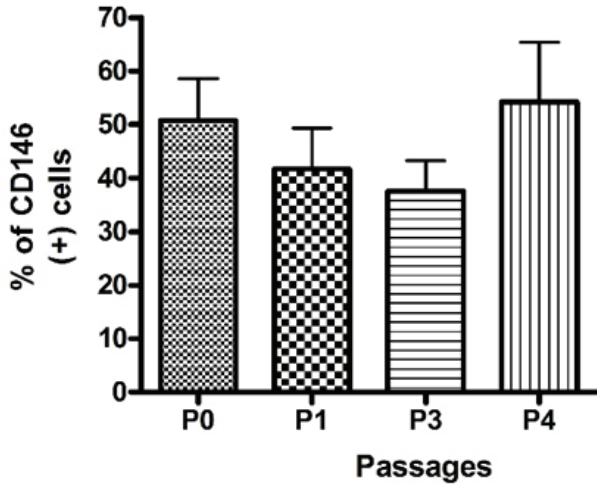

Figure 29 : Analyse de l'expression du récepteur CD146 (MCAM) durant les passages d'amplification in vitro.

Nous avons également choisi de nous intéresser au marqueur MSCA-1 (*Mesenchymal Stem Cell Antigen-1*), aussi appelé TNAP (*Tissue Non-specific Alkaline Phosphatase*)(Sobiesiak et al. 2010). *In vivo*, TNAP est exprimé dans la pulpe dentaire par les odontoblastes humains et dans la couche sous-odontoblastique (Figure 30)(Zweifler et al. 2014)(Tomlinson et al. 2015).

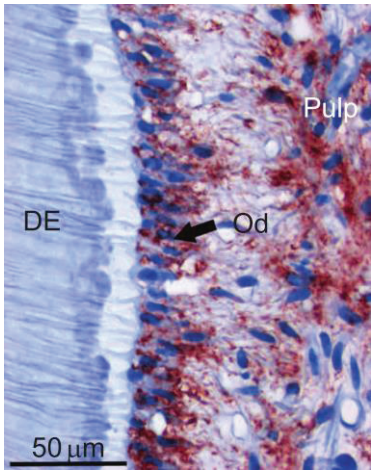

Figure 30 : Localisation de l'expression de la TNAP au niveau des odontoblastes (Od) bordant la dentine (DE) et des cellules de la région pulpaire sous-odontoblastique. (Zweifler et al. 2014)

TNAP est également exprimé par les ostéoblastes durant la phase de différenciation, mais diminue dans les cellules différenciées. Les CSM de la moelle osseuse, du périoste et de la gelée de Wharton expriment également ce marqueur qui a été décrit comme prédictif du caractère ostéogénique des cellules (Kim et al. 2012)(Olbrich et al. 2012)(Devito et al. 2014). Une étude récente réalisée avec des CPD humaines n'a toutefois pas permis de confirmer cette hypothèse (Tomlinson et al. 2015).

Nos premiers résultats indiquent que 5 à 10% des cellules présentes dans la pulpe dentaire *in vivo* expriment TNAP/MSCA-1, un pourcentage similaire à celui rapporté précédemment (Tomlinson et al. 2015). Lorsque des explants pulpaires sont mis en culture dans nos conditions, le pourcentage de cellules positives pour TNAP/MSCA-1 passe en moyenne à 15%, valeur qui reste stable pendant les 4 passages d'amplification (Figure 31).

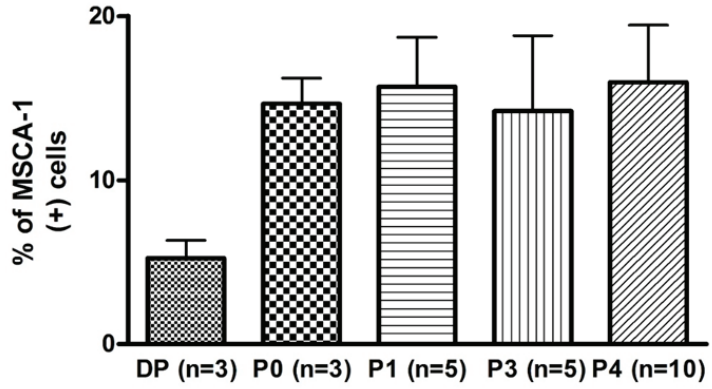

Figure 31 : Analyse de l'expression du récepteur MSCA-1(ou TNAP) durant les passages d'amplification in vitro.

Nous avons ensuite réalisé un tri cellulaire pour obtenir une population de cellules pulpaires enrichie en cellules TNAP/MSCA-1(+) (Figure 32).

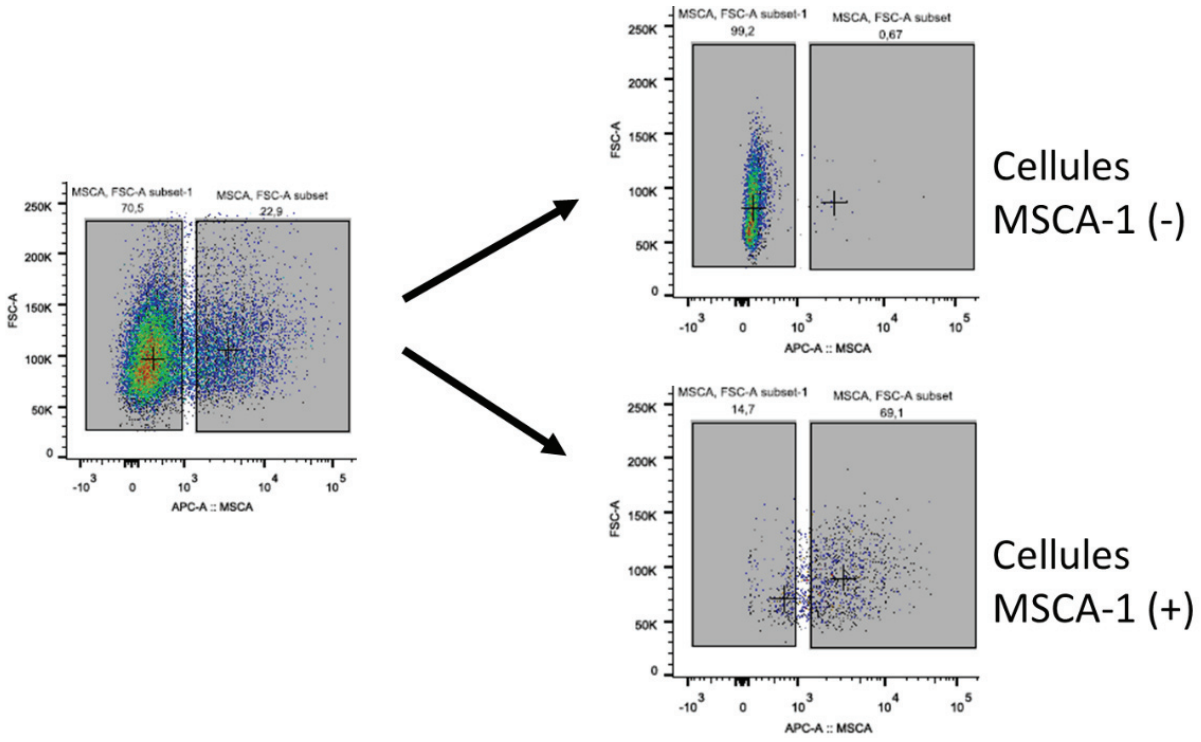

Figure 32 : Enrichissement réalisé grâce au tri cellulaire. Dans l'exemple choisi (CPD isolées in vitro et récupérées après 4 passages), les cellules MSCA-1(+) représentent 22,9% des cellules dans la population de départ. Après le tri cellulaire, la population MSCA-1(-) ne contient plus de cellules positives pour MSCA-1, alors que la population MSCA-1(+) contient plus de 69% de cellules positives pour ce marqueur.

Nous avons ensuite comparé le potentiel de différenciation ostéo/odontogénique de la population cellulaire de départ [MSCA-1(-/+)] avec les populations MSCA-1(-) et MSCA-1(+). L'analyse de la quantité de minéral déposé par les cellules après 4 semaines de culture (n = 2) indique que les cellules MSCA-1(+) ont un potentiel de minéralisation plus important que les cellules MSCA-1(-) (Figure 33). Ces résultats vont dans le sens de ceux obtenus avec d'autres populations de cellules stromales (Kim et al. 2012)(Olbrich et al. 2012)(Devito et al. 2014). Ils devront néanmoins être confirmés sur un nombre plus important d'échantillons. Ils diffèrent de ceux obtenus par Tomlinson et al. (2015) qui n'ont pas observé de différence de minéralisation entre les cellules de pulpe dentaire humaine MSCA-1(+) et MSCA-1(-) *in vitro* (Tomlinson et al. 2015). Cette différence de résultats pourrait être due à aux techniques d'isolement des cellules par dissociation, et de culture et d'amplification des CPD avec un milieu à base de sérum d'origine animale utilisées par ces auteurs. Si nos résultats sont confirmés, ils démontreront tout l'intérêt de notre protocole par rapport à ceux précédemment publiés.

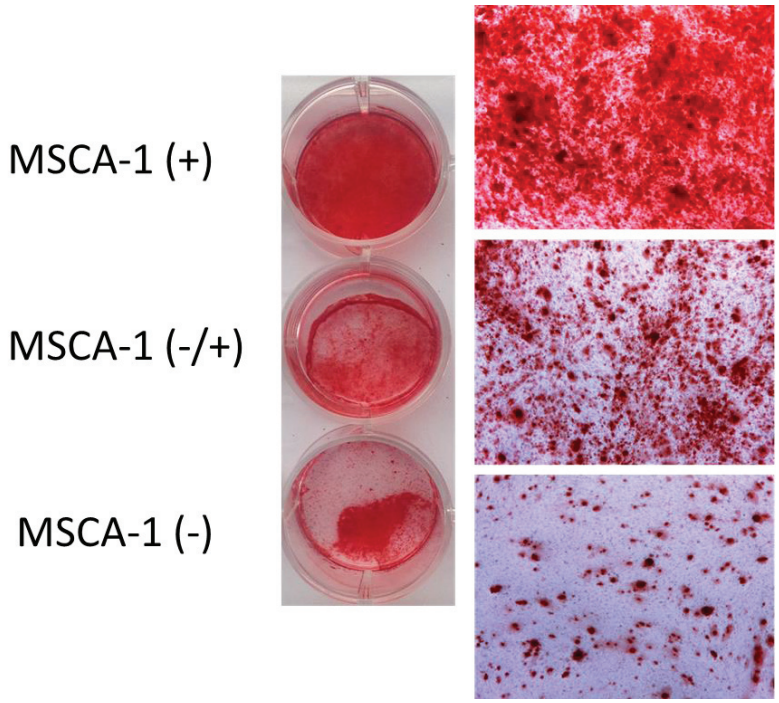

Figure 33 : Coloration à l'alizarine rouge de cellules pulpaires MSCA-1(+), MSCA-1(-) et MSCA-1 (80%(-)/20%(+) après trois semaines de culture en milieu de différenciation ostéo/odontogénique.

- **Régénération d'un complexe dentinopulpaire par des cellules souches/progénitrices de pulpe dentaire humaine ensemencées dans un biomatériau hybride fibrine/chitosane**

Projet lauréat de la Fondation des Gueules Cassées en 2015

Dans ce projet, nous souhaitons développer un biomatériau hybride innovant sous forme d'un hydrogel résorbable pour régénérer la pulpe dentaire humaine dans l'endodonte. Cet hydrogel servira de support à la formation d'un nouveau tissu qui va devenir progressivement une « pulpe équivalente ». Sa viscosité va être étudiée par rhéologie et la formulation optimisée pour permettre à l'hydrogel d'être facilement injectable dans l'endodonte. Les composants ont été choisis de telle sorte que le mélange final réticule sans ajout d'agent chimique, ce qui est un grand avantage en termes de cytotoxicité pendant les phases de gélification et de résorption de l'hydrogel. L'hydrogel sera fabriqué dans le cadre d'une collaboration avec le laboratoire d'Ingénierie des Matériaux Polymères de Lyon (UMR5223, Pr Laurent DAVID).

Lors d'une première phase, nous analyserons la viabilité des cellules pulpaires dans 4 formulations différentes de l'hydrogel, puis nous déterminerons également si les CPD ensemencées dans l'hydrogel modifient le volume (contraction) et la structure du biomatériau.

Lors d'une deuxième phase, nous nous placerons dans une perspective préclinique en implantant *in vivo* chez des souris immunodéficientes des fragments cylindriques de dentine radiculaire humaine dans lesquels aura été injecté le mélange CPD-hydrogel. L'extrémité coronaire de chaque fragment sera obturée par un biomatériau de reconstitution coronaire dentino-inducteur (Biodentine®) afin de mimer la situation clinique chez l'homme (Figure 34).

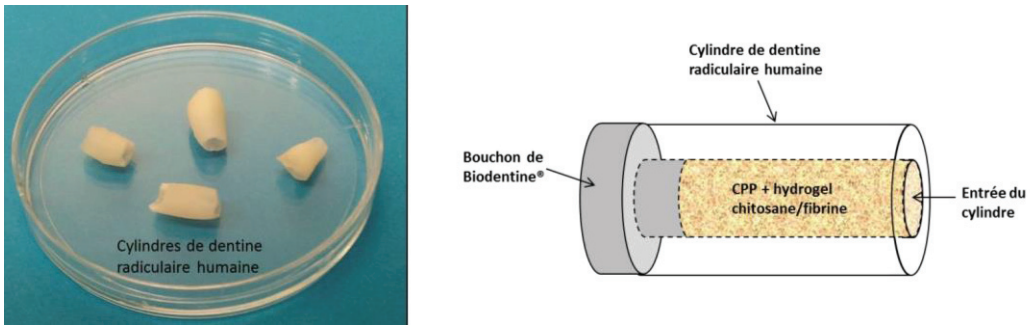

Figure 34 : Cylindre de dentine radiculaire humaine et représentation schématique du modèle de cylindre que nous utiliserons dans notre étude.

Après 1, 2, 4 et 6 semaines d'implantation, les souris seront euthanasiées, et les échantillons récupérés et analysés. Nous étudierons le comportement des CPD (prolifération et différenciation), la colonisation de l'hydrogel par les cellules de l'hôte, la cinétique de dégradation de l'hydrogel, ainsi que sa vascularisation et son innervation. Nous analyserons enfin la couche de dentine déposée par les CPD au contact de la Biodentine® et des parois dentinaires (Figure 35).

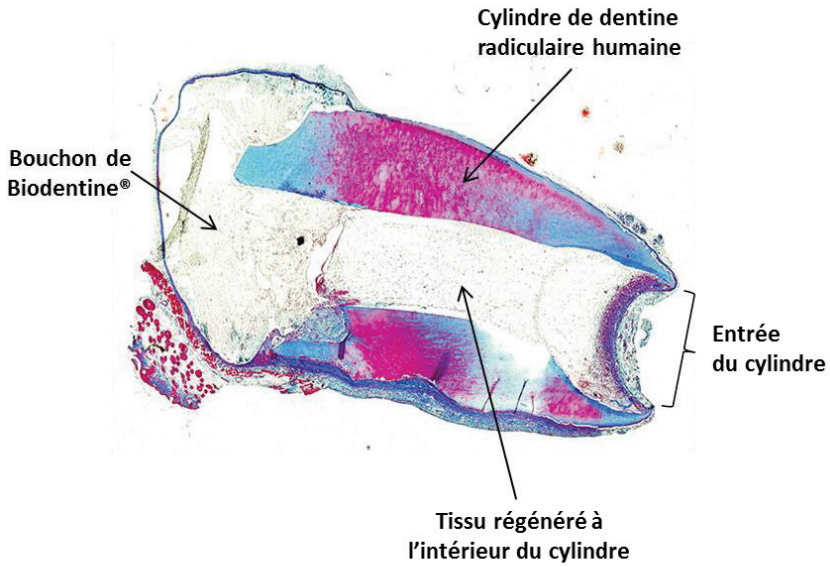

Figure 35 : Coupe histologique d'un fragment cylindrique de racine dentaire humaine ayant été implanté pendant 2 semaines sous la peau dorsale d'une souris immunodéficiente (Nude). L'exemple choisi montre que des cellules souches de tissu adipeux humain, ensemencées dans un gel de collagène de type I puis injectées dans le fragment radiculaire jusqu'au bouchon de Biodentine®, permettent la formation d'un tissu vascularisé dans la totalité de l'espace endodontique (Coloration : trichrome de Masson).

ANNEXES

Article 5 : Les cellules odontoblastiques produisent de l'oxyde nitrique avec une activité antibactérienne suite à l'activation du récepteur TLR2

FARGES J.-C., BELLANGER A., **DUCRET M.**, AUBERT-FOUCHER E., RICHARD B., ALLIOT-LICHT B., BLEICHER F., CARROUEL F.

Human odontoblast-like cells produce nitric oxide with antibacterial activity upon TLR2 activation

Front Physiol. 2015 6:185. doi: 10.3389/fphys.2015.00185

Résumé : La pénétration de bactéries orales cariogènes dans l'émail et la dentine durant le processus carieux déclenche une réponse immunitaire/inflammatoire dans le tissu pulpaire sous-jacent. La diminution de cette réponse est considérée comme un prérequis à la régénération du complexe pulpodentinaire. Si le rôle des odontoblastes dans la formation de la dentine est bien connu, leur implication dans la réponse antibactérienne face aux microorganismes cariogènes n'a pas encore été élucidée. Notre objectif a été de déterminer si les odontoblastes produisent de l'oxyde nitrique (NO) avec une activité antibactérienne suite à l'activation du récepteur Toll-like-2 (TLR-2), un récepteur de la membrane cellulaire impliqué dans la reconnaissance des bactéries cariogènes Gram-positive. Des cellules odontoblastiques humaines différenciées à partir d'explants de pulpe dentaire ont été stimulées avec un agoniste synthétique du TLR-2, Pam2CSK4. Nous avons découvert que l'expression des gènes codant pour NOS1, NOS2 et NOS3 est augmentée dans les échantillons stimulés par Pam2CSK4 par rapport aux échantillons non stimulés. *NOS2* est le gène le plus fortement stimulé. Les protéines NOS1 et NOS3 n'ont pas été détectées dans les cellules odontoblastiques contrôles ni dans les cellules stimulées. La synthèse de la protéine NOS2, l'activité globale des protéines NOS et le relargage de NO extracellulaire sont tous augmentés dans les échantillons stimulés. Le surnageant de culture des cellules stimulées avec Pam2CSK4 réduit la croissance de *Streptoccocus mutans*. Cet effet est contrebalancé par L-NAME, un inhibiteur spécifique des NOS. *In vivo*, l'expression du gène *NOS2* est augmentée dans les pulpes enflammées suite à une lésion carieuse par rapport aux pulpes saines. La protéine NOS2 est immunolocalisée dans les odontoblastes situés en regard de la lésion carieuse, mais pas dans les pulpes saines. Ces résultats suggèrent que les odontoblastes participent à la réponse pulpaire antimicrobienne destinée à lutter contre l'invasion de la dentine par les bactéries Gram-positives, en produisant du NO par l'intermédiaire de NOS2. Ils pourraient de cette manière préparer le terrain aux mécanismes de la cicatrisation et de régénération pulpaires.

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ORIGINAL RESEARCH
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Human odontoblast-like cells produce nitric oxide with antibacterial activity upon TLR2 activation

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The penetration of cariogenic oral bacteria into enamel and dentin during the caries process triggers an immune/inflammatory response in the underlying pulp tissue, the reduction of which is considered a prerequisite to dentinogenesis-based pulp regeneration. If the role of odontoblasts in dentin formation is well known, their involvement in the antibacterial response of the dental pulp to cariogenic microorganisms has yet to be elucidated. Our aim here was to determine if odontoblasts produce nitric oxide (NO) with antibacterial activity upon activation of Toll-like receptor-2 (TLR2), a cell membrane receptor involved in the recognition of cariogenic Gram-positive bacteria. Human odontoblast-like cells differentiated from dental pulp explants were stimulated with the TLR2 synthetic agonist Pam2CSK4. We found that *NOS1*, *NOS2*, and *NOS3* gene expression was increased in Pam2CSK4-stimulated odontoblast-like cells compared to unstimulated ones. *NOS2* was the most up-regulated gene. NOS1 and NOS3 proteins were not detected in Pam2CSK4-stimulated or control cultures. NOS2 protein synthesis, NOS activity and NO extracellular release were all augmented in stimulated samples. Pam2CSK4-stimulated cell supernatants reduced *Streptococcus mutans* growth, an effect counteracted by the NOS inhibitor L-NAME. *In vivo*, the *NOS2* gene was up-regulated in the inflamed pulp of carious teeth compared with healthy ones. NOS2 protein was immunolocalized in odontoblasts situated beneath the caries lesion but not in pulp cells from healthy teeth. These results suggest that odontoblasts may participate to the antimicrobial pulp response to dentin-invading Gram-positive bacteria through NOS2-mediated NO production. They might in this manner pave the way for accurate dental pulp healing and regeneration.

Keywords: tooth, odontoblast, caries lesion, dental pulp regeneration, nitric oxide, toll-like receptor 2, *Streptococcus mutans*

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Introduction

Odontoblasts are neural crest-derived, highly specialized mesenchymal cells organized as a densely packed layer at the periphery of the loose connective tissue situated in the center of the tooth, the dental pulp. Their main functions are the synthesis, extracellular deposition and mineralization of a collagen-rich matrix to form the dentin tissue that surrounds the dental pulp and underlies the surface enamel. Recent data have indicated that odontoblasts may also have functions not related to dentinogenesis (Bleicher et al., 2015). Indeed, because of their specific location at the pulp-dentin interface and the entrapment of their long cell process in dentin, they become exposed to dentin-invading oral bacteria during the carious process (Love and Jenkinson, 2002). Odontoblasts are the first cells encountered by tooth-invading pathogens and they have been suggested to initiate pulp immune and inflammatory responses to these pathogens (Durand et al., 2006; Veerayutthwilai et al., 2007). These responses may eliminate the insult and block the route of infection. Unchecked, bacterial invasion results in irreversible pulp inflammation then necrosis, and dissemination of potentially lethal microorganisms may occur throughout the body (Farges et al., 2009). In parallel, dentin formation appears greatly disturbed (Bjorndal and Mjör, 2001; Durand et al., 2006). Several lines of evidence now support the notion that it is only when pulp infection and inflammation are under control that dentinogenesis-based pulp regeneration will occur. In this context, further studies are needed to elucidate the odontoblast response to cariogenic bacteria in order to design new antibacterial therapeutics that will reduce dental pulp inflammation while promoting tissue healing and regeneration (Farges et al., 2013; Cooper et al., 2014).

Studies that aimed at elucidating the triggering of dental pulp immunity by odontoblasts have mostly focused on gram-positive bacteria, because these largely dominate the microflora in initial and moderate dentin caries lesions (Love and Jenkinson, 2002; Hahn and Liewehr, 2007). In particular, odontoblast-like cells were found to be responsive to lipoteichoic acid (LTA), a Gram-positive bacteria component recognized at cell surface through the pattern recognition receptor (PRR) Toll-like receptor-2 (TLR2). Engagement of odontoblast TLR2 by LTA up-regulates TLR2 itself and nucleotide-binding oligomerization domain 2, a cytosolic PRR. It also stimulates the production of the proinflammatory chemokines and cytokines CCL2, CXCL1, CXCL2, CXCL8, CXCL10, and interleukin (IL)-6, and the recruitment of immature dendritic cells (Durand et al., 2006; Staquet et al., 2008, 2011; Farges et al., 2009, 2011; Keller et al., 2010, 2011). IL-10, a cytokine that plays a central role in limiting host immune responses to pathogens, was also up-regulated. Similar, effects were observed when using Pam2CSK4, a synthetic diacylated lipopeptide analog that specifically binds TLR2. Of note, the response obtained with Pam2CSK4 was always higher than with LTA. However, no additional cytokine was induced by Pam2CSK4 compared to LTA, suggesting that odontoblasts secrete a limited set of proinflammatory factors when challenged with Gram-positive bacteria. We also observed that the lipopolysaccharide (LPS)-binding protein (LBP) was up-regulated by Pam2CSK4 (our unpublished results) and that it decreased TLR2 activation and proinflammatory cytokine production in odontoblast-like cells (Carrouel et al., 2013).

If the role of odontoblasts in the triggering and control of dental pulp immunity begins to be elucidated, their involvement in the direct fight against dentin-invading bacteria is far less known. Among antibacterial agents putatively produced by odontoblasts, nitric oxide (NO) has recently received particular attention. NO is a highly diffusible, gaseous free radical generated by nitric oxide synthases (NOS) through the conversion of L-arginine to L-citrulline. Three NOS isoforms have been identified so far: two are constitutively expressed at low levels, NOS1 (neuronal NOS) and NOS3 (endothelial NOS), whereas one is produced upon cell stimulation by microorganisms or proinflammatory cytokines, NOS2 (inducible NOS). NOS1 and NOS3 participate to normal tissue functions by constitutively synthesizing very small amounts (picomolar to nanomolar levels) of short acting NO (seconds to minutes). NOS2 is the enzyme responsible for high NO production in infection settings (Arthur and Ley, 2013). It is predominantly regulated at the transcriptional level and is involved in antibacterial defense by producing large amounts of NO, up to micromolar levels, for sustained periods of time (hours to days) (Nathan, 1992; Nussler and Billiar, 1993; MacMicking et al., 1997; Bogdan, 2001; Coleman, 2001; Guzik et al., 2003). Previous studies indicated that the *NOS2* gene was not or weakly expressed in healthy dental pulps, but was sharply up-regulated in inflamed ones (Law et al., 1999; Di Nardo Di Maio et al., 2004; Kawashima et al., 2005; Korkmaz et al., 2011). Human odontoblasts showed a marked immunoreactivity for 3-nitrotyrosine (a biomarker for NO-derived peroxinitrite) in inflamed pulp, suggesting that these cells release NO upon NOS2 activation. NO production by odontoblasts might be an important defense mechanism against dentin-invading oral microorganisms because it inhibits *Streptococcus mutans* growth (Silva-Mendez et al., 1999). Despite these important findings, no direct evidence was brought so far that odontoblasts produce NO amounts bactericidal for caries-related, gram-positive microorganisms. Therefore, the aim of our study was to investigate, in a culture model of human odontoblasts differentiated *in vitro*, if these cells are able to produce NO with antibacterial activity upon TLR2 engagement. We first studied the effects of Pam2CSK4 stimulation on odontoblast *NOS1*, *NOS2*, and *NOS3* gene expression. We also examined NOS1, NOS2, and NOS3 protein synthesis, NOS intracellular activity and NO secretion upon Pam2CSK4 stimulation. We then assessed the antibacterial effect of odontoblast-derived NO by analyzing the growth of *Streptococcus mutans* bacteria in the presence of TLR2-activated or control odontoblast-like cell culture supernatants. Finally, expression of NOS2 transcript and protein was investigated *in vivo* in healthy and bacteria-challenged inflamed dental pulps.

Materials and Methods

Reagents

The synthetic diacylated lipopeptide analog Pam2CSK4 was from InvivoGen (San Diego, CA, USA). The mouse

anti-NOS2 monoclonal antibody (clone 2D2-B2) was from R&D Systems Europe (Lille, France). Rabbit anti-NOS1 monoclonal (EP1855Y) and anti-NOS3 polyclonal antibodies were from Abcam (Cambridge, UK). The mouse anti-GAPDH monoclonal antibody (clone 8C2) was from Santa Cruz Biotechnology (Santa Cruz, CA USA). The mouse immunoglobulin G1 isotype control antibody (clone MOPC-21) and the arginine analog NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) were from Sigma-Aldrich (St Louis, MO, USA).

Dental Pulp Samples

Healthy and decayed human teeth were collected with informed consent of the patients or their parents, in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. Healthy pulps were taken from impacted third molars. Inflamed pulps were taken from decayed erupted molars with clinical features of acute pulpitis (deep dentin caries lesions, severe spontaneous dental pain for 12–24 h, no sensitivity to vertical or horizontal percussion, lack of periapical lesions) and in the absence of anti-inflammatory treatment.

Cell Culture and Treatments

Odontoblast-like cells were differentiated from dental pulp explants obtained from clinically healthy, impacted human third molars as previously described (Couble et al., 2000), then used for stimulation experiments. For detection of *NOS* gene expression, NOS protein production, NOS activity and NO extracellular levels, cells were cultured for the indicated times in the absence (controls) or in the presence of 10 µg/mL Pam2CSK4. To determine the antibacterial effect of odontoblast-like cell-derived NO on *Streptococcus mutans* bacteria, cells were treated with 10 µg/mL Pam2CSK4 for 24 h, and then culture media were collected and frozen until further use (see below). Some cultures were pretreated for 1 h with 0.5 or 1 mmol/L L-NAME prior to addition of Pam2CSK4 to confirm NO involvement in the effect observed.

Reverse Transcription-Polymerase Chain Reaction

RNA extraction and reverse transcription were performed from Pam2CSK4-stimulated and control odontoblast-like cells and from healthy and inflamed dental pulp samples as described (Keller et al., 2010; Farges et al., 2011). Real-time polymerase chain reaction (PCR) was performed in a CFX96 Real-Time PCR Detection System with the Fast Start Master SYBR Green I kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's specifications. The cyclophilin A housekeeping gene (*PPIA*) was used for sample normalization. Gene-specific primer sequences for *NOS1*, *NOS2*, *NOS3*, and *PPIA* are listed in **Table 1**. Annealing temperature was 65°C for all primer pairs. All runs were performed in duplicate. For each target gene, relative expression was determined after normalization using the Bio-Rad CFX Manager software. Results were expressed as fold change values relative to control odontoblast-like cell cultures for *in vitro* analyses and to healthy pulp samples for *in vivo* analyses.

Protein Extraction and Western Blotting

Cells were washed twice with PBS and overlaid with ice-cold RIPA buffer (Sigma-Aldrich) supplemented with a protease inhibitor cocktail (P8340, dilution 1:100; Sigma-Aldrich). After 5 min on ice, cells were scraped and insoluble material was removed by centrifugation at 12,000 g for 10 min at 4°C. Total proteins were quantified by a Bradford assay (Coomassie Protein Assay Reagent; Pierce, Thermo Fischer Scientific Inc., Rockford, IL, USA). Proteins were concentrated by precipitation for NOS isoform detection. After addition of 4 volumes of cold acetone and incubation for at least 1 h at −20°C, pellets were collected by centrifugation at 12,000 g for 10 min at 4°C, air-dried and dissolved in reducing Laemmli sample buffer. Identical amounts of proteins were loaded for Pam2CSK4-stimulated and control samples. Positive controls for NOS1 (neuronal NOS) and NOS3 (endothelial NOS) were extracts from mouse whole brain and human umbilical vein endothelial cells (HUVEC), respectively. HUVEC were kindly provided by Dr Laurent Müller (CIRB CNRS UMR7241 - INSERM U1050, Collège de France, Paris). Proteins were resolved by 8.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Molsheim, France). Membranes were probed with anti-NOS1 (diluted 1:1000), anti-NOS2 (1:400), anti-NOS3 (1:1000) or anti-GAPDH (1:2000) and incubated with HRP- or alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgGs (1:1000; Cell Signaling). Bound antibodies were detected on x-ray films by using Immunstar PA, Immunstar HRP or WesternC chemiluminescent substrates (Bio-Rad Laboratories).

Detection of NOS Activity and Extracellular NO Production

NOS activity and NO production by Pam2CSK4-stimulated and control odontoblast-like cells were quantified in cell lysates and culture media by using an Ultrasensitive Colorimetric NOS Assay kit and a Nitric Oxide Colorimetric Assay kit (Oxford Biomedical

TABLE 1 | Primers used for PCR analysis.

Gene	Forward primer	Reverse primer	Amplicon size (bp)
NOS1	CTGATACCAAAAGCCTCTCT	ATCTGAGCCTAACAATCTGG	76
NOS2	ACAGGCTCGTGCAGGACTCA	CACGGCTGGATGTCGGACTT	126
NOS3	CATGAGCACTGAGATCGGCA	CCAGGATGTTGTAGCGGTGA	59
PPIA	GGATTGCTTGAGCCTAGAGTGA	CCTCTGCCTACCTTTGAGACAC	87

Research, Oxford, MI, USA), respectively, according to the manufacturer's specifications. Cells were lysed in Beadlyte® Cell Signaling Universal Lysis Buffer (Millipore). NO is highly labile, with a half-life in the order of seconds, and these kits are based on the colorimetric quantitation of nitrite (NO_2^-), the stable end product of NO oxidation, using Griess reagent. They include the conversion of nitrate to nitrite by NADH-dependent enzyme nitrate reductase before nitrite measurement, in order to provide for accurate determination of total NO production. Absorbance values were read at 540 nm using a microplate reader. NO/nitrite sample concentrations were determined from a sodium nitrite standard curve. Intracellular NO/nitrite concentration was expressed as μmoles/μg of proteins (determined using a Bradford protein assay).

Streptococcus mutans Growth Assessment

The *Streptococcus mutans* strain (CIP 103220) was purchased from Institut Pasteur (Paris, France). Bacteria were cultured in Brain Heart Infusion broth (Biomérieux, Marcy-L'Etoile, France) at 37°C for 24 h. After centrifugation at 2500 rpm for 5 min, the supernatant was discarded and the bacterial pellet resuspended in water for determination of the bacteria number with McFarland Standard (Biomérieux). Samples containing 10^8 bacteria were then taken and centrifuged at 15,000 rpm for 5 min. Bacterial pellets were resuspended in 100 μL odontoblast-like cell culture supernatants, then maintained at 37°C for 15, 30, 60, or 90 min, the later time being just before the end of the bacteria growth phase as determined by our preliminary experiments (not shown). Bacteria-containing samples were then plated onto Columbia agar supplemented with 5% defibrinated horse blood. Bacterial cultures were performed in anaerobic conditions (GENbox anaer, Biomérieux) for 5 days at 37°C, then *S. mutans* colonies were counted.

Immunohistochemistry

Healthy teeth and carious ones with inflamed pulps were fixed in 4% paraformaldehyde-phosphate-buffered saline solution for 7 days, demineralized in 10% acetic acid for 4 months, and routinely treated for paraffin embedding. Eight-micrometer serial sections were then cut, deparaffinized, and rehydrated. Endogenous peroxidase was blocked by incubation in 0.3% hydrogen peroxide for 15 min at room temperature. Sections were incubated for antigen retrieval in 10 mmol/L citrate buffer (pH 6.0, 98°C) for 10 min and then blocked with normal horse serum for 45 min at room temperature. Sections were then incubated with 12.5 μg/mL anti-NOS2 antibody overnight at 4°C. Staining controls were performed by using a mouse immunoglobulin G1 isotype. Antibody detection was performed by using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol, peroxidase being localized with diaminobenzidine.

Statistical Analysis

Results were expressed as mean values ± standard deviation obtained from different odontoblast-like cell cultures or tooth pulps originating from human third molars obtained from different donors. Statistical analysis was performed with a paired *t*-test. A $p < 0.05$ was considered significant.

Results

Pam2CSK4 Increases *NOS1*, *NOS2*, and *NOS3* Gene Expression and NOS2 Protein Synthesis in Odontoblast-Like Cells

We previously found that the synthetic lipoprotein analog Pam2CSK4 at a 10 μg/mL concentration was particularly efficient to activate TLR2 in odontoblast-like cells and induced the production of proinflammatory cytokines and chemokines (Keller et al., 2010, 2011; Farges et al., 2011; Staquet et al., 2011). Therefore we used, in the present study, the same concentration to assess Pam2CSK4 effect on the expression of *NOS1*, *NOS2*, and *NOS3* genes by these cells. We observed that Pam2CSK4 significantly up-regulated the three genes tested (**Figure 1A**). The gene coding for the inducible NOS, *NOS2*, was the most up-regulated one, the increase being maximal after 8 h of cell stimulation. At the protein level, NOS2 was strongly increased after 8 h of Pam2CSK4 stimulation compared to control unstimulated cells (**Figure 1B**). NOS1 and NOS3 were not detected in control or Pam2CSK4-stimulated samples stimulated for 8 or 16 h (not shown).

Pam2CSK4 Increases NO Production by Odontoblast-like cells

To determine whether NOS2 up-regulation upon Pam2CSK4 stimulation was accompanied by an increase in NOS activity and NO production, intracellular and extracellular NO concentrations were assessed by the measure of the NO degradation end-product nitrite. We observed a strong intracellular nitrite increase that was maximal after 8 h (**Figure 2A**). To assess NO diffusion to the extracellular compartment, we measured nitrite concentration in supernatants of Pam2CSK4-stimulated odontoblast-like cells. We found a progressive accumulation of nitrite in the culture medium reaching a concentration of approximately 40 μmol/L after 24 h, the longest stimulation time tested (**Figure 2B**). In unstimulated samples, the nitrite concentration remained low and constant with time, of approximately 10 μmol/L.

Odontoblast-Like Cell-Derived NO Reduces *Streptococcus mutans* Growth

Next, to evaluate the NO effect on the growth of cariogenic microorganisms, odontoblast-like cells were stimulated or not with 10 μg/mL Pam2CSK4 for 24 h with or without pretreatment with the NOS inhibitor L-NAME. Culture supernatants were collected and placed into contact with *Streptococcus mutans* bacteria for 15, 30, 60, or 90 min. In unstimulated samples, pretreatment with L-NAME increased the number of *Streptococcus mutans* colony-forming units, suggesting that NO from unstimulated odontoblast-like cells limits *Streptococcus mutans* growth (**Figure 3**). We observed that the number of *Streptococcus mutans* colony-forming units was clearly reduced in odontoblast-like cells stimulated with Pam2CSK4 compared to unstimulated ones, indicating a stronger antibacterial effect of culture supernatants from TLR2-activated cells. The decrease in NO production owing to increasing concentrations of L-NAME in Pam2CSK4-stimulated samples led to an augmentation of

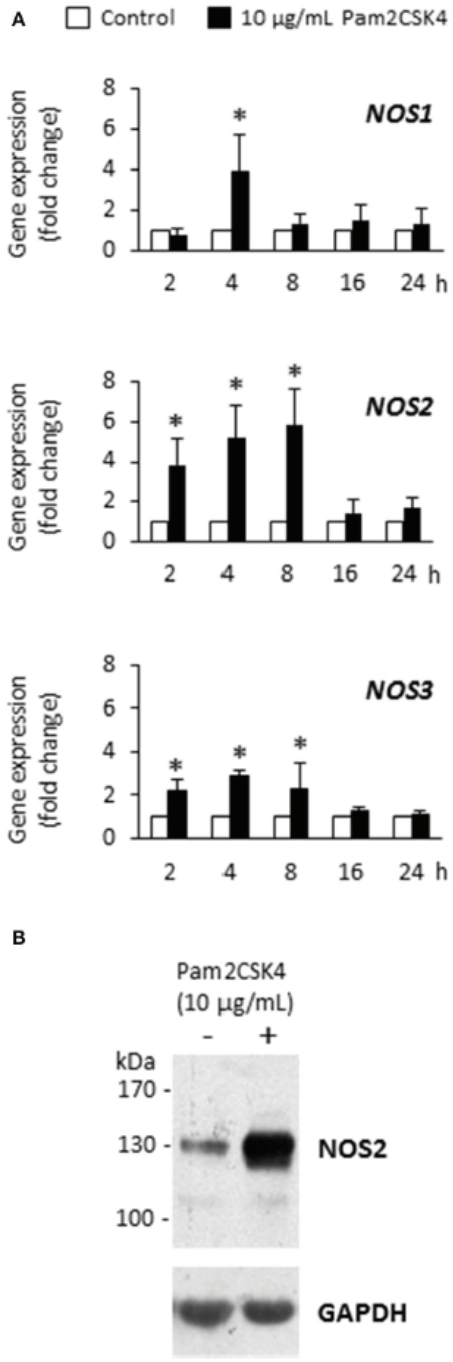

FIGURE 1 | Pam2CSK4 increases *NOS1*, *NOS2*, and *NOS3* gene expression and NOS2 protein synthesis in odontoblast-like cells. (A) Analysis of *NOS1*, *NOS2*, and *NOS3* gene expression after cell challenge with 10 µg/mL Pam2CSK4 for the indicated times. The three genes were significantly up-regulated upon Pam2CSK4 stimulation, *NOS2* being the most up-regulated one ($n = 4$). *$p < 0.05$. **(B)** At the protein level, NOS2 synthesis was clearly increased after 8 h of odontoblast-like cell stimulation with 10 µg/mL Pam2CSK4. The gel shown is representative of three independent experiments.

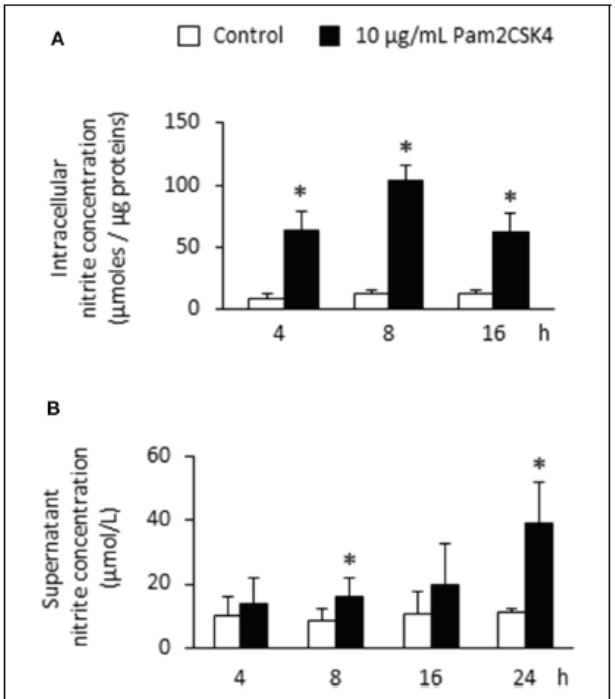

FIGURE 2 | Pam2CSK4 increases NOS activity and extracellular NO production by odontoblast-like cells. (A) Analysis of intracellular NO by the measurement of nitrite concentration in cells stimulated with 10 µg/mL Pam2CSK4 for the indicated times. Production of intracellular nitrite was strongly increased in PAM2CSK4-stimulated samples, being maximal after 8 h ($n = 5$). **(B)** Determination of NO concentration in culture supernatants of odontoblast-like cells stimulated with 10 µg/mL Pam2CSK4. Nitrite progressively accumulated in the culture medium, reaching a concentration of approximately 40 µmol/L after 24 h of stimulation ($n = 5$). *$p < 0.05$.

the number of *Streptococcus mutans* colony-forming units, thus indicating that NO production by odontoblast-like cells was indeed responsible for the observed slowdown of bacterial growth.

NOS2 Transcript and Protein are Up-Regulated in Inflamed Pulps from Decayed Teeth

To assess the *in vivo* relevance of these findings and determine whether NOS2 is expressed in odontoblasts, NOS2 transcript and protein were examined in healthy dental pulps and inflamed samples from carious teeth. We found that the *NOS2* gene was strongly up-regulated in inflamed pulps compared to healthy ones (**Figure 4A**). NOS2 protein was clearly detected by immunostaining in odontoblasts and subodontoblast cells in the inflamed area, but not in cells in the non-inflamed area far from the lesion (not shown) or in healthy pulps (**Figure 4B**). Staining controls performed by using the mouse immunoglobulin G1 isotype were negative.

Discussion

Odontoblasts situated at the dental pulp periphery could play a role in the initiation of the tissue defense against

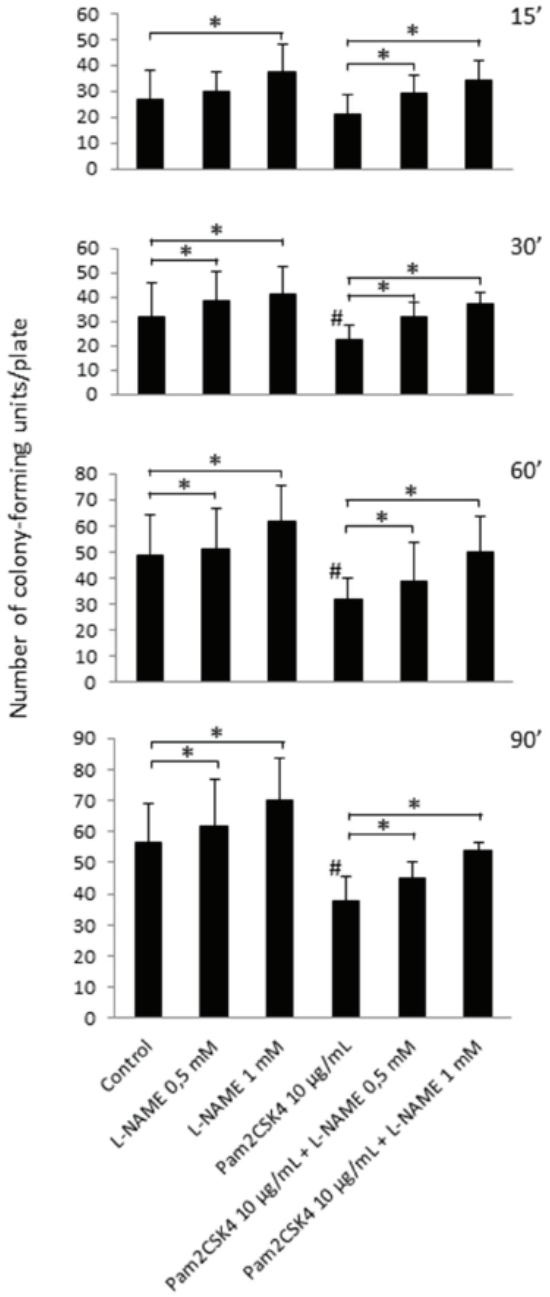

FIGURE 3 | NO released from unstimulated or Pam2CSK4-stimulated odontoblast-like cells alters *Streptococcus mutans* viability. Analysis of *Streptococcus mutans* growth after contact of bacteria for 15, 30, 60, or 90 min with culture supernatants of cells challenged with 10 µg/mL Pam2CSK4 for 24 h in the presence or in the absence of L-NAME. The number of *Streptococcus mutans* colony-forming units was reduced when odontoblast-like cells were stimulated with Pam2CSK4 compared to unstimulated ones. Pretreatment with the NOS inhibitor L-NAME increased the number of *Streptococcus mutans* colony-forming units in both unstimulated and Pam2CSK4-stimulated samples ($n = 6$). $^{\#}p < 0.05$ vs. control unstimulated cells. $^{*}p < 0.05$ vs. cultures without L-NAME.

dentin-invading Gram-positive oral bacteria through their ability to recognize pathogens and to produce proinflammatory cytokines and chemokines (Farges et al., 2013). In this context, chemokine secretion from odontoblasts may induce accumulation of immune cells at the pulp-dentin interface, including antigen-presenting immature dendritic cells, to uptake bacterial by-products diffusing through dentin tubules and evolve pathogen-specific innate and adaptive responses (Durand et al., 2006). However, whether odontoblasts are able to directly destroy intradentinal or peripheral pulp-reaching microorganisms by producing antibacterial agents, without the intervention of immune cells, it is largely unknown. Nitric oxide is a highly diffusible, antimicrobial free radical generated and released by many cell types in inflammatory conditions to kill or inhibit the replication of a variety of microorganisms. NO antimicrobial activity is primarily due to NO reactivity with superoxide anion to form highly cytotoxic peroxynitrite, S-nitrosylation of thiol residues that changes protein conformation, inactivation of enzymes by disruption of iron centers, DNA damage, and membrane lipid peroxidation (Guzik et al., 2003). In this report we provide evidence that odontoblasts differentiated *in vitro* from human dental pulp explants are able to synthesize large amounts of NOS2 and produce NO with antibacterial activity upon TLR2 activation. We first observed that *NOS1*, *NOS2*, and *NOS3* gene expression was significantly increased in Pam2CSK4-stimulated cells compared to controls, *NOS2* being the most up-regulated gene. In agreement with this finding, NOS2 protein was clearly up-regulated in Pam2CSK4-stimulated cells, suggesting its involvement in NO production by these cells. Conversely, NOS1 and NOS3 isoforms, although clearly found in brain and endothelial cell extracts, respectively, were not detected in our Western blot analysis in stimulated or control samples. Accordingly, we speculate that most, if not all, of NO production by Pam2CSK4-stimulated odontoblast-like cells is due to the induction of NOS2, as shown in macrophages stimulated *in vitro* with lipopolysaccharide, another pattern recognition receptor ligand (Denlinger et al., 1996; MacMicking et al., 1997; Guzik et al., 2003).

We found that NO was produced to levels (several tenths of micromoles/liter) similar to those observed in macrophages *in vitro* stimulated with LPS or *Staphylococcus aureus* LTA, or with an association of *Streptococcus mutans* LTA and IFN-γ (Denlinger et al., 1996; Matsuno et al., 1998; Hong et al., 2014). This indicates that the responsivity of odontoblasts upon TLR2 activation, in terms of NO production, is comparable to that of specialized immune cells. To our knowledge, NO concentrations have not been reported in inflamed pulps. However, those we measured in Pam2CSK4-stimulated odontoblast-like cell supernatants were in the range of the concentrations found in periapical exudates from infected human root canals (Shimauchi et al., 2001). This indicated that NOS2-dependent NO amounts produced by TLR2-activated odontoblast-like cells is biologically relevant. We observed here that the number of *Streptococcus mutans* colony-forming units was significantly reduced by NO in stimulated odontoblast-like cells compared to control ones, suggesting the role of NO produced by odontoblasts in the fight against cariogenic bacteria.

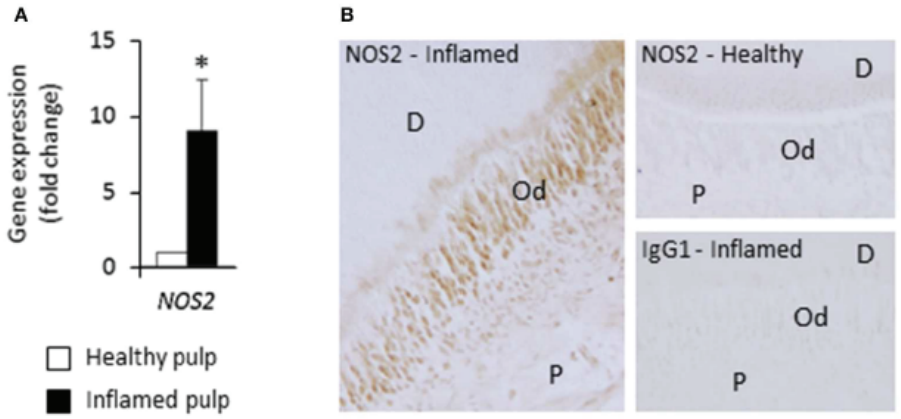

FIGURE 4 | NOS2 transcript and protein are up-regulated in inflamed pulps from decayed teeth compared to healthy ones. (A) Analysis of *NOS2* gene expression in healthy and inflamed pulps with real-time RT-PCR ($n = 3$). $^*p < 0.05$. **(B)** Immunohistochemical localization of NOS2 protein in healthy and bacteria-challenged inflamed pulps. No NOS2 staining was found in healthy sample, but odontoblasts and subodontoblast cells were stained in inflamed pulp beneath the caries lesion. D, dentin; Od, odontoblast layer; P, pulp. Data shown are representative of results obtained from independent experiments performed with two healthy and two carious teeth.

We then detected immunohistochemically the NOS2 protein in odontoblasts of inflamed pulps situated beneath dentin caries lesions, whereas it was not present in odontoblasts from healthy ones. This result is in accordance with the fact that NOS2 is not expressed in resting cells, but is only synthesized upon cell activation (Coleman, 2001). Odontoblasts from healthy teeth were also found to be negative for NOS2 (Kawanishi et al., 2004; Kawashima et al., 2005; Mei et al., 2007). NOS2 synthesis was increased in inflamed pulps beneath caries lesions or when inflammation was experimentally induced with bacterial by-products (Kawanishi et al., 2004; Kawashima et al., 2005; Mei et al., 2007; Korkmaz et al., 2011), underscoring the importance of this enzyme in NO production in pulp inflammatory conditions. We observed NOS2 immunoreactivity in odontoblast and subodontoblast cells of the peripheral inflamed pulp, as previously reported in human teeth (Korkmaz et al., 2011). This indicates that odontoblasts are not the only cells involved in the fight again dentin-invading microorganisms. Other cells might also include leukocytes, as previously shown (Guzik et al., 2003). We do not exclude that NOS1 and NOS3 could contribute to the high NO production in the odontoblast layer of bacteria-challenged inflamed pulps. However, several data suggests that such a contribution, if any, would be limited since NOS1 and NOS3 are only able to produce very low concentrations of NO (nanomolar range) compared to NOS2 (micromolar) (Coleman, 2001). The results presented here support the view that NOS2 is a key player in the production of physiologically relevant NO amounts by odontoblasts in bacteria-challenged inflamed pulps. NO could have deleterious effects on odontoblasts themselves. However, these effects might be limited since soluble guanylate cyclase, the receptor that mediates NO effects inside the cell, is decreased in odontoblasts in inflamed human pulps compared to healthy ones (Korkmaz et al., 2011). This indicates that the NO present in the odontoblast layer would rather act on neighboring cells and/or, as suggested by the present study, on dentin-invading bacteria. In this context, NO production by odontoblasts might modulate neurotransmission from deep dentin up to nerve endings present in the proximal dentin or the pulp periphery and/or might contribute to the regulation of the vascular tone of adjacent vessels. The large amount of NO synthesized by odontoblast NOS2 under pathological conditions might thus have an analgesic effect but might also dilate local blood vessels (McCormack and Davies, 1996; Di Nardo Di Maio et al., 2004).

NO has been recently proposed for a clinical use in the treatment of periodontal diseases, because of its drastic reduction of the viability of periodontopathogens (Backlund et al., 2014). Our results also demonstrated an antibacterial effect for NOS2-dependent production of NO by odontoblast-like cells. In the caries context, evidence of NO effects in animal models with cariogenic bacteria is warranted before envisaging the clinical use of NO to prevent dentin and pulp tissue colonization and subsequent irreversible pulpitis and necrosis.

Finally, studies have shown that NO can induce the differentiation of several cell types including osteoblastic, neuronal and endothelial cells (Beltran-Povea et al., 2015). It may also play a part in odontoblast differentiation and the subsequent formation of reparative dentin, notably by augmenting osteocalcin synthesis and alkaline phosphatase activity (Mei et al., 2007; Yasuhara et al., 2007). So NO donors could be use in regenerative dentistry as adjuvants to promote the mineralization events that lead to dentin or bone tissue formation. Additional studies using our culture model and others are required to test this hypothesis.

In summary, we report for the first time that NO with antibacterial activity is produced by TLR2-activated human odontoblast-like cells. Further studies are needed to determine the potential beneficial effect of this molecule on the reduction of human dental pulp inflammation and the promotion of tissue healing and regeneration.

Acknowledgments

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(O : oral ; P : poster)

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- Invitées

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O. DUCRET M.
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Ingénierie tissulaire de la pulpe dentaire : vers le développement d'un nouveau médicament de thérapie innovante

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Ces dernières années, des thérapies à base de cellules mésenchymateuses ont été développées pour améliorer les thérapies qui visent à réparer l'homme et notamment la pulpe dentaire. Dans ce contexte, la dent apparait comme la source de cellules mésenchymateuses, souches ou progénitrices, permettant de réparer la pulpe dentaire. En effet, la pulpe dentaire est facile d'accès et les cellules pulpaires présentent un fort potentiel de différenciation.

Actuellement, les différents organismes de contrôle recommandent d'utiliser des procédures standardisées pour l'isolement, le stockage et l'expansion des cellules en culture pour garantir une sécurité et une reproductibilité optimale lorsque les cellules sont utilisées en culture cellulaire. Cependant, la plupart des procédures utilisées pour la production de cellules à partir de la pulpe dentaire ne sont pas entièrement satisfaisante, car elles peuvent altérer les propriétés biologiques et la qualité des cellules. En effet, les procédures d'isolement cellulaire, d'enrichissement, de cryopréservation et d'amplification pendant de nombreux passages dans des milieux contenant des produits d'origine animale ou humaine sont connues pour affecter le phénotype des cellules, la viabilité, la prolifération et les capacités de différenciation.

Ce travail de thèse s'intéresse à compiler les stratégies actuelles de fabrication de produits cellulaires à partir de la pulpe dentaire, puis il propose de nouveaux protocoles pour améliorer l'efficacité, la reproductibilité et la sécurité de ces nouvelles stratégies thérapeutiques. Ainsi nous avons isolé, amplifié et cryopréservé des cellules de la pulpe dentaire. Grace à un travail d'immunophénotypage, nous avons pu étudier différentes sous-populations à l'intérieur de la population totale. Enfin nous avons montré que ces cellules sont capables de rester congelées pendant plus de 500 jours sans présenter d'anomalies du caryotype et de conserver un potentiel de différenciation ostéo/odontogénique.

Nos perspectives de travail sont aujourd'hui de développer un modèle de régénération pulpaire *in vivo* chez le petit animal, dans un environnement tridimensionnel permettant aux cellules de survivre, d'adhérer, de proliférer et de se différencier en cellules pulpaires.

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